

**Immunological abnormalities in patients with haemophilia;
role of factor VIII concentrate**

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DECLARATION

In accordance with the regulations of the University of Edinburgh I declare that this thesis was composed by me and the work reported is my own except for those portions specified in the acknowledgements at the end of the text.

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ABSTRACT

Intermediate purity factor VIII concentrates have greatly improved care for haemophilia sufferers but have been implicated in contributing to subclinical immunomodulation observed *in vivo*. During the course of factor VIII replacement therapy haemophilia patients are repeatedly exposed to an enormous number of allogeneic protein contaminants and this substantial protein load may be responsible for establishing immunological abnormalities. Indeed, it has been proposed that intermediate purity factor VIII concentrates could accelerate the rate of disease progression in those haemophiliacs infected with the human immunodeficiency virus. Highly purified factor VIII preparations are now available commercially, but are extremely expensive compared to conventional intermediate purity concentrates and their use must therefore be justified in terms of patient benefit.

This study sets out to investigate whether intermediate purity factor VIII concentrates could contribute to immune abnormalities observed in haemophilia patients *in vivo*. This problem has been approached by investigating the effects of various factor VIII preparations on immune stimulation *in vitro*.

A range of factor VIII preparations were included in a number of proliferation assays. Peripheral blood lymphocytes were stimulated with mitogens, recall antigen or allogeneic stimulator cells. The effect of factor VIII concentrates on lymphocyte proliferation was monitored by recording ^3H -thymidine incorporation, cytokine production or cell surface activation marker expression post stimulation.

The results demonstrate that intermediate purity factor VIII concentrates can modulate lymphocyte proliferation *in vitro*. SNBTS intermediate purity factor VIII preparations inhibited lymphocyte proliferation following PHA stimulation but enhanced lymphocyte activation following stimulation with irradiated allogeneic lymphoblastoid cells. In contrast, highly purified factor VIII preparations had no effect on immune stimulation following a number of activation signals. Although the factor(s) responsible for the observed immune modulation *in vitro* have not been identified, several potential candidates have been excluded. The immunomodulatory effect of some intermediate

purity factor VIII preparations *in vitro* may partially account for immunological imbalance observed in haemophilia patients.

In lymphocytes infected with HIV, intermediate purity factor VIII concentrate has been shown to enhance lymphocyte proliferation and virus replication following stimulation with irradiated allogeneic stimulator cells. Factor VIII infusions could potentially accelerate disease progression in HIV infected haemophilia patients by a similar mechanism to that observed *in vitro*.

The use of highly purified factor VIII preparations in the treatment of haemophilia may be justified.

	Page
TITLE PAGE	1
DECLARATION	2
ABSTRACT	3
CONTENTS	5
ABBREVIATIONS	12
INTRODUCTION	14
1 Factor VIII replacement therapy	14
2 The immune system	16
3 Blood components and immunosuppression	16
3.1 Immunological abnormalities in haemophilia patients	
(i) Immune abnormalities <i>in vivo</i>	16
(ii) Immune abnormalities <i>in vitro</i>	19
3.2 Immunological abnormalities in patients receiving blood transfusions	22
3.3 Graft survival in renal transplant patients	23
3.4 Cancer recurrence rates	24
3.5 Tumour survival in animals	24
3.6 Foetal tolerance in pregnancy	25

4	Virus infection in haemophilia patients	26
5	Patients receiving Scottish National Blood Transfusion Service intermediate purity factor VIII concentrate	27
5.1	Immunological abnormalities	27
5.2	The Edinburgh haemophilia cohort	28
5.3	Immunological abnormalities relating to HIV infection	29
5.4	HIV disease progression	30
5.5	SNBTS intermediate purity factor VIII concentrate	31
6	Factor VIII concentrate and immune modulation	32
6.1	Effect of factor VIII preparations on lymphocyte activation	
	(i) Lymphocyte proliferation	32
	(ii) IL2 production	33
	(iii) Activation marker expression	34
6.2	Effect of factor VIII preparations on monocyte function	34
6.3	Effect of factor VIII preparations on NK cell function	35
	AIMS	36
	MATERIALS AND METHODS	38
1	Cells	38
1.1	Peripheral blood mononuclear cells	38
1.2	Aminoethylthiuronium bromide-treated sheep red blood cells	38
1.3	Separation of T lymphocytes	39
1.4	Lymphoblastoid cell lines	39

2	Lymphocyte proliferation assays	39
2.1	Mitogen stimulation	39
2.2	Antigen stimulation	40
2.3	One-way mixed lymphocyte reactions	40
2.4	Labelling cells with ^3H -thymidine	41
2.5	Harvesting cells	41
2.6	Reproducibility of data	41
3	Human immunodeficiency virus (HIV) infection	41
3.1	HIV infection of lymphocytes	41
3.2	HIV inactivation	41
3.3	HIV infected lymphocyte proliferation assays	42
3.4	Viral quantification	42
4	Cytokine assays	43
4.1	Conditioned medium	43
4.2	Maintaining CTLL-2 cells	43
4.3	Supernatants tested for IL2 activity	43
4.4	IL2 bioassay	43
5	Flow cytometry	44
5.1	Indirect immunofluorescence	44
5.2	Expression of cell surface activation markers	44
5.3	Expression of HLA.DR	45
5.4	Two colour flow cytometry	45
6	Factor VIII	46
6.1	Source	46
6.2	Tissue culture	48

6.3	Cell Viability	48
6.4	Defibrinating SNBTS factor VIII	48
6.5	Physical manipulation of SNBTS factor VIII	49
6.6	Selective protein depletion	49
7	Fibronectin	50
7.1	One-way mixed lymphocyte reactions	50
8	Polyacrylamide gel electrophoresis	50
8.1	Sample preparation	50
8.2	Polyacrylamide gels	50
8.3	Gel staining	
	(i) Coomassie blue staining	51
	(ii) Silver staining	51
	RESULTS	52
1	Effects of factor VIII concentrate on lymphocyte proliferation following stimulation with phytohaemagglutinin	52
1.1	³ H-thymidine incorporation	52
1.2	Cell surface activation marker expression	53
1.3	IL2 production	53
1.4	Preincubation of SNBTS Z8 factor VIII with peripheral blood mononuclear cells	54
1.5	Filtered SNBTS factor VIII concentrate	54
2	Effect of SNBTS factor VIII batch Z8-6026 on lymphocyte proliferation following stimulation with tetanus toxoid	54

3	Effect of SNBTS factor VIII batch Z8-6026 on lymphocyte proliferation following stimulation with pokeweed mitogen	55
4	Effect of factor VIII concentrate on lymphocyte proliferation following stimulation with irradiated allogeneic lymphoblastoid cells	55
4.1	³ H-thymidine incorporation	55
5	Characterisation of cells in mixed lymphocyte reactions	56
6	Physical manipulation of SNBTS intermediate purity factor VIII concentrate	56
6.1	Freezing, filtering and defibrinating factor VIII	56
6.2	Molecular weight cut off filters	57
7	Fibronectin	57
8	Preincubation of SNBTS Z8 factor VIII with peripheral blood mononuclear cells or irradiated lymphoblastoid cells	57
9	Incubation of SNBTS Z8 factor VIII with fresh, fixed, or irradiated lymphoblastoid cells	58
10	SDS-PAGE	58
10.1	Composition of factor VIII concentrates	58
10.2	Electrophoretic profile following filter fractionation	59
11	Selective protein depletion	59
12	SNBTS factor VIII and HLA.DR expression in lymphoblastoid cells	60

13	SNBTS factor VIII and cell viability	60
14	Mixed lymphocyte reactions and culture media	61
15	Lymphocyte proliferation assays and HIV infection	61
15.1	PHA stimulation	61
15.2	One-way mixed lymphocyte reactions	61
16	Mixed lymphocyte reactions in haemophilia patients	62
16.1	HIV antibody negative	62
16.2	HIV antibody positive	62
	DISCUSSION	64
1	Effects of factor VIII concentrate on lymphocyte proliferation following stimulation with phytohaemagglutinin	64
2	SNBTS batch Z8-6026	68
3	Effects of factor VIII concentrate on lymphocyte proliferation following stimulation with irradiated allogeneic lymphoblastoid cells	69
4	Lymphocyte proliferation, factor VIII and HIV infection	73
5	Lymphocyte proliferation in haemophilia patients	75
	REFERENCES	79

APPENDIX I	96
APPENDIX II	97
APPENDIX III	98
ACKNOWLEDGEMENTS	99

ABBREVIATIONS

AET	aminoethylthiuronium bromide
AIDS	acquired immunodeficiency syndrome
BPL	Bio Products Laboratory, Elstree
BSA	bovine serum albumin
CMV	cytomegalovirus
Con A	concanavilin A
DTT	dithiothreitol
DNCB	dinitrochlorobenzene
FCS	foetal calf serum
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HSV-2	herpes simplex virus type-2
ICAM	intercellular adhesion molecule
Ig	immunoglobulin
IL	interleukin
IFN	interferon
MHC	major histocompatibility complex
NK	natural killer cell
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHA	phytohaemagglutinin
PMA	phorbol-myristate-acetate

PMN	polymorphonuclear cells
PPD	purified protein derivative of tuberculin
PWM	pokeweed mitogen
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNBTS	Scottish National Blood Transfusion Service, Edinburgh
SRBC	sheep red blood cells

INTRODUCTION

1 Factor VIII replacement therapy

Factor VIII deficiency or haemophilia A is a hereditary bleeding disorder confined almost entirely to males. A reduction in factor VIII activity in the blood coagulation causes uncontrollable haemorrhage which must be stopped by the intravenous administration of factor VIII.

Historically whole blood or fresh frozen plasma have been used as a source of replacement factor VIII. However, in 1964 it was discovered that factor VIII could be partially purified by precipitation in the cold and subsequently cryoprecipitate became the chosen form of therapy (Pool et al, 1964). In the early 1970s lyophilized factor VIII concentrate was introduced and has remained in use to date (Shanbrom and Thelin, 1969). Recently high purity factor VIII concentrates have been developed using ion-exchange chromatography or monoclonal antibodies to purify factor VIII from human plasma (Burnouf et al, 1991, Brettler et al, 1989, Weinstein, 1989). In addition, preliminary clinical trials using synthetic factor VIII prepared by recombinant DNA technology are under way (White et al, 1989, Schwartz et al, 1990). These highly purified but costly preparations are however not yet widely available to the majority of haemophilia sufferers in the United Kingdom.

Factor VIII concentrates have substantially improved the ease and effectiveness of substitution therapy but they have also introduced transfusion-associated complications (Aronson, 1988, Johnson et al, 1985).

Factor VIII concentrate is manufactured from very large pools of human plasma (eg. 5-30,000 donations) and thus carries the risk of transmitting a number of blood-borne viruses. Lyophilized factor VIII concentrate has been associated with the transmission of hepatitis B virus, hepatitis C virus, parvo virus and human immunodeficiency virus (Norkrans et al, 1981, Fletcher et al, 1983, Mortimer et al, 1983, Williams et al, 1990, Simmonds et al, 1990, Kitchen et al, 1984, Jones et al, 1985).

Following the inadvertent exposure to HIV and infection of a large number of haemophiliacs, in 1984 virus inactivation steps were introduced into the manufacturing process of factor VIII concentrates. Approaches to virus inactivation vary from wet or dry state heating (Spire et al, 1985, Levy et al, 1985, Schimpf et al, 1989) to ultraviolet irradiation in conjunction with beta propiolactone (Prince et al, 1983) and treatment with organic solvents and detergents (Horowitz et al, 1985, Feinstone et al, 1983). Heat treatment results in factor VIII concentrates of comparable therapeutic value to unheated concentrates although there is some loss (approximately 10-20%) of factor VIII activity during the viral inactivation procedures (McGrath et al, 1985).

Most factor VIII used in the treatment of haemophilia A patients is of intermediate purity. In some cases as little as one percent of the lyophilized product is actually factor VIII, the vast majority of material being fibrinogen, immunoglobulin, fibronectin and other plasma proteins. Although some protein contaminants are not antigenic in their native state, manufacturing procedures may lead to the formation of aggregated or denatured proteins capable of stimulating the immune system. In particular, new antigenic epitopes may be created during heat treatment or other virus inactivation steps. Haemophiliacs may also be exposed to alloantigens such as erythrocyte blood group antigens during the course of their treatment.

The method of heating factor VIII concentrate can alter the polypeptide structure of factor VIII (Kemball-Cook et al, 1990). In particular, pasteurization (60°C for 10 hours) leads to an increase in factor VIII breakdown as does heating lyophilized powder in organic solvent suspension (60°C for 20 hours) rather than ordinary dry heating (60°C for 36 hours). Although some protocols for virus inactivation result in increased factor VIII protein degradation, it is not clear whether product immunogenicity is altered in parallel. To date there is no evidence to support this possibility.

Allain et al (1985) compared the biochemical and immunological characteristics of a heated factor VIII concentrate with an identical non-heated preparation. Rabbits were inoculated intradermally with either the heated or non-heated factor VIII product. Antisera raised were tested by double immunodiffusion in agarose gel against both concentrates, fibronectin and fibrinogen. Precipitin line profiles were identical for heated and non-heated preparations as immunogen suggesting the heat-treated product contained no new antigenic determinants.

It has been postulated that the huge antigenic load patients are exposed to may modulate

the haemophilic immune system in some manner. It is possible that factor VIII concentrates of variable composition and purity produced by different manufacturing processes may affect the immune system to differing extents.

2 The immune system

The human immune system comprises a complex inter-related network of organs, cells and specialised proteins including antibodies, cytokines and complement. Following antigenic challenge cells of the immune system interact to respond appropriately in a controlled, co-ordinated fashion.

Antigen presenting cells at various sites throughout the body internalise, process and present antigen on their cellular membranes. Antigen fragments are presented in the context of host major histocompatibility antigens. Antigens are presented to helper T lymphocytes which are central to the development of immune responses. They can enhance the activity of B cells to produce antibody and that of cytotoxic T cells, via the production of lymphokines. Activated helper T cells may also modulate the activity of NK cells, macrophages, granulocytes and antibody-dependent cytotoxic cells.

Antigen presenting cells not only present antigen fragments, but produce a number of cytokines essential to the regulation and response of the immune system (Hildemann, 1984). It is clear that interference with any component part of the immune system could potentially disrupt the entire immune response. Intermediate purity factor VIII concentrates may therefore affect general immune status via modulation of lymphocyte and monocyte function.

3 Blood components and immunosuppression

A growing body of data supports the proposal that blood transfusions have immunological effects on patients and experimental animals beyond those of alloimmunisation to blood cell antigens (Woodruff and van Rood, 1983).

3.1 Immunological abnormalities in haemophilia patients

(i) Immune abnormalities *in vivo*

It has been clear for a number of years that some haemophilia patients undergoing

treatment with factor VIII preparations have subclinical abnormalities of the immune system. Patients tend to have reduced T helper:T suppressor/cytotoxic ratios due to depressed numbers of CD 4⁺ cells and in some cases increased numbers of CD 8⁺ cells (Saidi, 1983, Menitove, 1983, Lederman, 1983, Lechner, 1983, Kessler, 1983, Jones, 1983).

In 1982 a number of haemophilia patients were found to be suffering from the acquired immunodeficiency syndrome, AIDS (Centers for Disease Control, 1982). However, immunological abnormalities were present in many haemophilia patients in the absence of full blown AIDS. At this time it was not clear whether immune modulation in haemophiliacs was related to viral infection by a putative AIDS virus, some feature of nonA nonB hepatitis, or indeed a consequence of intensive factor VIII concentrate usage. Although it became clear that HIV, the virus responsible for AIDS, could be transmitted via the transfusion of blood products and many haemophiliacs were indeed infected with HIV (Centers for Disease Control, 1986), a role for factor VIII replacement therapy in establishing immune imbalance in haemophilia patients was not completely excluded.

More recent studies have confirmed that immunological abnormalities occur in patients suffering from haemophilia, irrespective of HIV infection. Reduced CD 4⁺ cell numbers, increased CD 8⁺ cell numbers and reduced CD4:CD8 ratios all occur in HIV seronegative haemophilia patients receiving factor VIII concentrates (Carr et al, 1984, Berntorp et al, 1989, Rickard et al, 1983, Moffat et al, 1985, Gill et al, 1985, AIDS-Haemophilia French Study Group, 1985). Depressed CD4:CD8 ratios have also been reported in anti-HIV negative haemophiliacs treated with cryoprecipitate (Ceuppens et al, 1984, Hollan et al, 1985).

The cell mediated immune response may be assessed by the intradermal injection of standard doses of recall antigens. Haemophilia A patients challenged with a panel of recall antigens show impaired reactivity, irrespective of HIV antibody status (McIvor et al, 1987). Madhok et al (1986) evaluated the cell mediated immune response in a group of haemophiliacs from Glasgow by means of the dinitrochlorobenzene (DNCB) skin test. Cell mediated immunity was depressed in both HIV positive and HIV negative patients. In addition, an inverse relationship between exposure to clotting factor concentrate and DNCB skin response was observed.

Immune abnormalities unrelated to HIV infection are readily demonstrable in haemophilia patients. However, abnormalities may be a result of haemophilia per se or a consequence of intensive factor VIII replacement therapy with blood products.

The significance of subclinical immune imbalance in haemophiliacs is unclear. Altered CD4:CD8 ratios may indicate abnormal regulation of the immune response or some other defect in T cell mediated immunity. However, the composition of lymphocyte subsets in blood is affected by a number of conditions. Altered CD4:CD8 ratios have been reported in an enormous number of disease states (Westermann and Pabst, 1990). Reduced responsiveness to recall antigens following intradermal challenge may be related to a defect in T cell or antigen presenting capabilities.

The clinical consequences of subtle immunological abnormalities in haemophilia patients seem to be relatively limited. Madhok et al (1987) report a rare case of extranodal non-Hodgkin's lymphoma in a haemophilia patient seronegative for HIV. The authors propose that reduced cell mediated immunity may have contributed to the pathogenesis of the tumour in this patient.

One might predict that patients with reduced cell mediated immunity would be more susceptible to viral infections, tumours and intracellular bacterial infections (Hildemann, 1984).

Chronic persistent hepatitis is more likely to progress to more serious forms of liver disease in haemophilia patients than in non haemophiliacs with this condition (Triger and Preston, 1990). Progression to chronic active hepatitis and even cirrhosis has been recorded within as little as five years. It is difficult to determine whether severe liver disease in haemophilia patients is related to increased susceptibility to hepatitis virus infection or merely a result of virus exposure. Although a single episode of infection can result in chronic persistent hepatitis, repeated exposure and/or infection may compound the severity and progression of liver disease.

Rosendaal et al (1989) calculated mortality figures for a group of 717 Dutch haemophiliacs between 1973 and 1986. When compared to the general male population, cancer mortality was significantly higher (2.5 times) than expected. More than half the excess cancer mortality was caused by lung tumours. Ischaemic heart disease was significantly reduced in this group of patients. Increased cancer mortality

is also suggested in a report by Aronson et al (1988).

There have been no reports of an increased incidence of lymphoma in haemophilia patients. However, in a study of AIDS-associated non-Hodgkin lymphoma in the USA, lymphoma was found to be most common in patients with haemophilia or clotting disorders (Beral et al, 1991).

Deaths in haemophilia patients in the United Kingdom from 1976 to 1980 were analysed by Rizza and Spooner (1983). Although the study reports a near normal median life expectancy in patients with severe haemophilia, it also highlights an unusually high incidence of deaths from pneumonia in patients suffering from haemophilia A.

However, these data should be interpreted with caution. In this study, the incidences of deaths from various causes were calculated on the basis of death certificate analysis. It is possible that pneumonia may not have been the only, or principal cause of death, but may have been recorded on the death certificate nevertheless. Pneumonia may arise as a complication of underlying disease such as following a stroke due to intracranial haemorrhage.

Haemophiliacs appear to be unusually susceptible to Mycobacterium tuberculosis infection (Beddall et al, 1983). In an outbreak of the disease in hospital, the frequency of pulmonary tuberculosis in boys with bleeding disorders was found to be significantly greater than in patients with other paediatric conditions. The incidence of pulmonary tuberculosis in haemophiliac boys was similar to that in immunocompromised children receiving cytotoxic chemotherapy for leukaemia and solid tumours. Furthermore, there was a significant correlation between the amount of factor VIII replacement therapy received and the development of evidence of tuberculosis infection.

(ii) Immune abnormalities *in vitro*

A number of studies have evaluated immune function *in vitro* using peripheral blood mononuclear cells from haemophiliacs.

Both peripheral blood lymphocytes and CD 4⁺-enriched T cells show reduced responsiveness to the non-specific T cell mitogen PHA in both HIV seropositive and HIV seronegative patients. In contrast, responses of the haemophiliac to CMV and HSV-2 viral recall antigens do not significantly differ from those of healthy control subjects (Mahir et al, 1988).

It is unlikely that low proliferative responses to PHA *in vitro* are due to excessive

suppressor T cell activity as CD 8⁺ depleted T cell cultures from haemophilia patients do not respond normally to PHA stimulation (Mahir et al, 1988).

Haemophiliacs have a reduced capacity to produce interleukin 2 following PHA mitogen stimulation *in vitro* (Madhok et al, 1990). Impaired IL2 production is independent of HIV-1 antibody status, mean annual dose of factor VIII clotting concentrate administered and liver disease severity. Similarly, peripheral blood mononuclear cells from multitransfused haemophiliacs have a diminished capacity to produce IFN- γ in response to induction with PHA, irrespective of HIV antibody status (Ruffault et al, 1988).

T cell proliferation in response to autologous Escherichia coli-pulsed monocytes is significantly decreased in patients with haemophilia as compared to healthy controls. This defect is not associated with exposure to HIV and not related to annual factor VIII usage (Mannhalter et al, 1986). It is not clear whether aberrant antigen processing and presentation or impaired T cell reactivity is responsible for the observed poor response. Monocytes from haemophilia patients certainly seem to be abnormal. Sternbach et al (1987) report alterations in monocyte function including impaired chemotaxis, increased Candida pseudotropicalis killing and reduced adhesion to plastic.

The infusion of factor VIII preparations can modulate levels of circulating immune complexes and affect the mononuclear phagocytic system *in vivo*. In a small series, Kimberly et al (1984) reported that although individual patient responses to factor VIII infusion varied, there was a correlation between changes in mononuclear phagocyte function and circulating immune complexes.

Pasi and Hill (1990) investigated monocyte phagocytic function in six haemophiliac boys before and one hour after factor VIII infusion. Five of the boys were anti-HIV seronegative. Monocyte phagocytic function was assessed by the ingestion of anti-Rh(D) sensitized O Rh(D) positive erythrocytes and was significantly impaired following factor VIII infusion.

Repeated infusions of factor VIII concentrate caused monocyte phagocytic function to become more impaired. Recovery to normal baseline levels of monocyte phagocytic function occurred as treatment was reduced. The effect of repeated factor VIII infusion on monocyte function was investigated in only three haemophiliac boys. Although two of the boys were undergoing surgery, the third had not. Similar patterns of inhibition were observed for each boy. Surgery per se was therefore not solely responsible for

reduced phagocytic function.

Immunological abnormalities in haemophilia patients are not confined to T lymphocytes and monocytes. Polymorphonuclear cells from haemophilia patients have significantly impaired chemotactic, phagocytic and killing capabilities when compared to normal controls. PMN functions are diminished regardless of HIV infection (Antonaci et al, 1988).

HIV seronegative haemophiliacs have impaired proliferative responses to Staphylococcus aureus. Immunoglobulin secretion by plaque-forming cells *in vitro* following stimulation with PWM is also reduced (Gorski et al, 1986). However, spontaneous immunoglobulin production *in vitro* is elevated in haemophilia patients when compared to normal, healthy controls (Jin et al, 1989). Similarly, serum IgG levels are abnormally high in haemophiliacs. Some haemophiliacs also appear to have elevated titres of autoantibodies directed against both nuclear and cytoplasmic specificities (Matheson et al, 1987).

The observed B cell abnormalities in haemophilia patients are consistent with chronic B cell activation *in vivo*. B cell abnormalities appear to be unrelated to the amount or type of factor VIII replacement therapy received. In fact, B cell abnormalities are present, although less profound, in the small number of haemophilia patients who have received very little or no factor VIII substitution therapy (Jin et al, 1989). Similarly, NK cell activity is low in haemophilia patients regardless of whether or not they have received blood products (Porzsolt et al, 1984). The abnormality is not correlated with HIV exposure. It is probable that the NK cell defect is functional as the number of cells bearing NK cell markers in haemophiliacs is normal (Jin et al, 1989). NK cells from haemophilia patients show impaired responsiveness to IFN- β and IFN- γ (Matheson et al, 1986).

It is possible that immune dysfunction in haemophiliacs may be genetically determined rather than a consequence of blood product exposure. This has not to date been convincingly demonstrated.

Larger, more complete studies of "virgin" or previously untransfused haemophilia patients are required before a genetic predisposition to immunological imbalance can be confirmed or refuted. This type of study is, of course, very difficult to perform.

Most patients with severe haemophilia, who might be expected to have the most profound abnormalities, are diagnosed at six to twelve months of age when the immune system in normal babies is immature.

3.2 Immunological abnormalities in patients receiving blood transfusions

Gascon et al (1984) reported immunological abnormalities in patients receiving multiple blood transfusions. Patients included in the study group suffered from sickle cell disease, beta thalassemia and sideroblastic and idiopathic refractory anemias. NK cell function was severely depressed in transfusion recipients.

Reduced NK cell activity was not related to the underlying disease process and was apparently a result of transfusion. NK cell activity was normal in patients suffering from sickle cell disease who had never been transfused or who were no longer receiving transfusions. Indeed, the degree of NK cell activity was inversely related to the amount of blood transfused.

Although CD4:CD8 T cell ratios were normal in this group of patients, virtually all patients receiving regular packed erythrocyte transfusions had markedly elevated levels of HLA-DR positive T cells. Furthermore, HLA-DR expression was abnormal in patients with a history of transfusion who were no longer receiving red blood cells. This suggests that the administration of packed erythrocyte transfusions can lead to chronic T cell activation.

It must be noted that the HIV antibody status of patients included in this study is unknown and some immunological abnormalities may be related to underlying HIV infection.

The screening of all blood donations for antibody to HIV (then termed human T-lymphotropic virus type III) in the United States was not introduced until 1985. Early in 1985 the frequency of units of blood collected which were consistently positive for HIV specific antibodies was 0.25% (Leads from the MMWR, 1985). Patients with thalassemia major and sickle cell disease who receive transfusions of packed erythrocytes have abnormally high levels of serum IgG and β_2 -microglobulin (AIDS-Hemophilia French Study Group, 1985). However, this may reflect chronic hepatitis rather than immune modulation due to transfusion of blood products alone.

3.3 Graft survival in renal transplant patients

The administration of blood transfusions prior to renal transplant is beneficial to kidney graft survival. Opelz et al (1978) demonstrate a striking correlation between the number of pretransplant blood transfusions and improved transplant survival.

The mechanism responsible for improved allograft survival is unknown, although it is conjectured that the establishment of immunological tolerance or non-specific immunosuppression may play a role.

Transplantation studies in animals support a role for blood transfusions in establishing immune tolerance.

In a prospective study of kidney allograft survival in rhesus monkeys, animals receiving five consecutive pretransplant blood transfusions showed a four-fold increase in mean survival time compared to non-transfused controls (van Es et al, 1977).

Patients on regular haemodialysis prior to renal transplantation have suppressed cell mediated immunity, as assessed by a quantitative DNCB skin test. Weak DNCB reactors have a significantly higher graft survival rate when compared to strong DNCB reactors (Watson et al, 1979). The number of blood transfusions received is correlated with reduced response to DNCB (Schot and Schuurman, 1986).

Fischer et al (1980) investigated the effect of blood transfusions on cell mediated immunity in previously nontransfused prospective kidney graft recipients. Patients received leukocyte-poor blood transfusions and their cellular immunity was assessed by recording lymphocyte responses to mitogen and antigen stimulation *in vitro*. A marked suppression of cellular immunity which was more pronounced and prolonged following a second transfusion was demonstrated. The authors propose transfusion-induced suppression of cell mediated immunity may be mediated by a non-specific suppressor cell. Similarly, Klatzmann et al (1983) describe nonspecific suppression to allogeneic cells generated *in vivo* by blood transfusion.

It is unclear whether depressed cell mediated immunity following the administration of blood transfusions is long lasting or indeed sufficient to cause clinically recognisable immune deficiency. One might predict that such immune suppression could result in an

increased incidence of tumours or other malignant disorders in organ transplant recipients. Investigating the frequency of such diseases in transplant recipients is complicated by their dependence on immunosuppressive drug regimens which ensure continued graft survival.

3.4 Cancer recurrence rates

A number of studies have shown an association between perioperative blood transfusions and cancer recurrence rates in patients undergoing cancer surgery. Patients suffering from various tumours, including cancer of the colon, rectum, lung, breast and cervix in addition to soft tissue sarcomas are reported to have significantly higher rates of recurrence following transfusion (Burrows and Tartter, 1982, Blumberg et al, 1985, Foster et al, 1985, Tartter et al, 1984, Hyman et al, 1985, Tartter et al, 1985, Blumberg et al, 1985, Rosenberg et al, 1985). However, most of these studies were carried out retrospectively by analysing medical records and are therefore subject to biases. In particular, it is probable that patients with unfavourable prognostic factors were more likely to receive more blood transfusions at the time of surgery.

Blumberg et al (1986) present data from patients with colonic, rectal, cervical and prostate tumours. A proportional hazards risk analysis showed that transfusion of any whole blood or more than three units of erythrocytes was significantly associated with earlier recurrence and death due to cancer. It was concluded that some factor present in greater amounts in whole blood, such as plasma, may contribute to the increased risk of recurrence in patients who have undergone transfusion. Presumably reduced cell mediated immune surveillance or NK cell activity resulting from blood transfusion allows the spread of malignant cells to proceed unchecked.

3.5 Tumour survival in animals

In a rat model, Francis and Shenton (1981) investigated the effects of blood transfusion on the growth of transplanted chemically induced sarcoma. Transplants grew more rapidly in rats that had received transfusions of allogeneic blood than in rats that had received syngeneic blood, or had not been transfused.

The growth of transplanted tumours in mice is promoted by transfusions involving

different H-2 antigens. The effect is especially evident when transfusions of whole blood, plasma, or lymphocytes are administered. Transfusions of erythrocytes alone do not result in increased tumour growth (Horimi et al, 1983).

3.6 Foetal tolerance in pregnancy

The clinical consequences of blood product transfusion are consistent with the hypothesis that transfusion of blood in some way results in generalized immune suppression.

Allogeneic components of the major histocompatibility complex may trigger an immunoregulatory mechanism which results in immune suppression. Indeed, the immunosuppressive events following parenteral or anal MHC alloantigenic challenge have been likened to maternal immunosuppression during pregnancy (Hoff and Peterson, 1989). Whereas immunosuppression of pregnancy is generally localized to the uterus, parenteral or anal exposure to MHC alloantigens results in systemic immune suppression.

Although a mother is exposed to foreign foetal MHC antigens in early pregnancy, the allogeneic foetus is not rejected. This is in stark contrast to the rejection of MHC incompatible transplantation grafts (Medawar, 1944).

The degree of foetal alloantigenic challenge depends on how similar maternal and paternal HLA haplotypes are. Paradoxically, the greater the MHC alloantigenic challenge, the more favourable a pregnancy is likely to be in terms of reduced risk of spontaneous abortion, foetal growth retardation and maternal morbidity (Mogil and Wegmann, 1989).

In some instances, couples with unexplained infertility or increased rates of spontaneous abortion share more HLA antigens than is usual (Komlos et al, 1977, Gerencer et al, 1979). In these circumstances infertility can be overcome by transfusing leukocyte-enriched plasma throughout pregnancy (Taylor and Page Faulk, 1981).

In some pregnant women, the suppressive immune response to paternally inherited foetal alloantigens is not localized to the maternofoetal interface. McMichael and Sasazuki (1977) describe how lymphocytes from a multiparous woman fail to respond

to allogeneic lymphocytes from her husband in a mixed lymphocyte reaction. The effect is due to antigen specific T suppressor cells.

Antilymphocyte immunoglobulins found in sera from multiparous women (Payne and Rolfs, 1958, van Rood et al, 1958) are specific for both foetal and paternal lymphocytes. Maternal γ -globulin concentrate prepared from clotted blood in placental tissue recovered post partum, is immunosuppressive to kidney transplant patients. Renal graft survival rate is improved following transfusion of maternally-derived γ -globulin concentrate (Riggio et al, 1978).

It is not known if immune suppression subsequent to blood product transfusion and foetal tolerance during pregnancy are related. However, exposure to MHC alloantigens may well play a crucial role in both phenomena.

4 Virus infection in haemophilia patients

There can be no doubt that immunological abnormalities are present in haemophilia sufferers. It is however, difficult to state with certainty that immune abnormalities arise because of blood product exposure.

Very few groups have been able to study the effects of factor VIII concentrate in the absence of confounding effects from HIV infection, chronic liver disease or infection with hepatitis C or hepatitis B viruses. Each of these factors may well interact to complicate the situation further. For instance, it is possible that intermediate purity factor VIII infusions in some way alter HIV disease progression. Immunosuppressive concentrates might make HIV infected haemophilia patients more susceptible to opportunistic infection. Alternatively, immunostimulatory products could enhance virus replication thus contributing to more rapid HIV disease progression. Similarly, it has been suggested that HIV-induced immunosuppression may allow nonA nonB hepatitis or hepatitis B infections to proceed unchecked (Madhok et al, 1990).

It seems unlikely that all immune abnormalities are secondary to liver disease. Both haemophilia A and haemophilia B patients suffer from similar liver problems, yet haemophilia A patients tend to have more profound immunological abnormalities when compared to haemophilia B patients. Haemophilia A patients use more units of factor VIII activity than haemophilia B patients use factor IX. In addition, factor VIII preparations contain more protein contaminants than factor IX concentrates, which tend

to be of greater purity.

Studies assessing the immunological status of previously untransfused patients, who have received only heat treated or high purity factor VIII are required to clarify this situation.

Evans et al (1991) have recently presented a study of this type on fifteen haemophilic boys who were treated exclusively with heat treated BPL 8Y concentrate. This preparation has not been associated with the transmission of hepatitis B virus, HIV or hepatitis C virus (Pasi and Hill, 1989, Skidmore et al, 1990). Preliminary results suggest that treatment with 8Y concentrate does not lead to altered CD 4⁺ or CD 8⁺ T cell numbers. However, the boys have been observed for under three years and should be monitored prospectively for some time. BPL 8Y concentrate has approximately ten times more factor VIII activity per mg of protein compared to SNBTS Z8 concentrate.

If immune abnormalities *in vivo* are a consequence of immunosuppression by intermediate purity factor VIII concentrates, the introduction of highly purified preparations may reduce such immune modulation. However, the absence of concentrate-induced immunosuppression could theoretically lead to increased factor VIII inhibitor formation. Although early inhibitor development in association with the use of highly purified factor VIII has been reported (Bell et al, 1990), the true incidence of inhibitory antibodies remains controversial.

5 Patients receiving Scottish National Blood Transfusion Service intermediate purity factor VIII concentrate

5.1 Immunological abnormalities

In the Spring of 1983 a study assessing the immunological status of haemophilia patients attending the Edinburgh Haemophilia Centre was undertaken (Carr et al, 1984). The original purpose of the study was to investigate modifications in the immune system related to haemophilia itself and the effect of therapeutic factor VIII concentrates on immunological parameters.

Patients included in the study had been treated exclusively with factor VIII and factor IX products manufactured from blood donations collected locally by the Scottish National

Blood Transfusion Service. At that time no case of AIDS had been reported in Scotland and it was therefore assumed that the haemophilia patients under investigation were not latently infected with the (then) putative AIDS virus. Immunological abnormalities demonstrated in this study group were therefore attributed to the infusion of factor VIII concentrates per se rather than infection by the human immunodeficiency virus. Retrospective testing of stored serum samples has confirmed that the patients were anti-HIV negative at the time of the study.

Immunological alterations were observed in both haemophilia A and haemophilia B patients. Haemophilia A patients demonstrated reductions in absolute total lymphocyte and total T cell counts. The T lymphocytopenia resulted from a reduction in T helper cells. T suppressor/cytotoxic cell counts were normal. Approximately half the haemophilia A patients had reduced CD4:CD8 ratios. A small group of haemophilia B patients included in the study also had depressed CD4:CD8 ratios. This was due to a slight decrease in the mean T helper cell number and a slight increase in suppressor/cytotoxic cell numbers. There was no correlation between abnormalities of circulating lymphocyte subsets and severity of haemophilia, amount of substitution therapy received, abnormalities of liver function or immunoglobulin concentrations. Cell mediated immunity in this group of patients was assessed in terms of reactivity to intradermally injected recall antigens. Patients receiving SNBTS factor VIII concentrate had depressed intradermal delayed type hypersensitivity responses. The degree of responsiveness to recall antigens was inversely related to mean annual factor VIII consumption (Ludlam, 1988). This implies that SNBTS factor VIII concentrate has a suppressive effect on cell mediated immunity.

5.2 The Edinburgh haemophilia cohort

On follow-up, a number of haemophiliacs in the Edinburgh study group were found to have developed antibodies to HIV-1 (Ludlam et al, 1985). A few patients had received commercial factor VIII, whilst others had previously been treated with cryoprecipitate. However, transfusion records indicated that the majority of HIV infected patients had received factor VIII from a single batch of contaminated SNBTS factor VIII concentrate. Blood for this batch of factor VIII was collected in 1983 when HIV infection in the Scottish blood donor population was very low. It is therefore possible that haemophilia

patients transfused with this batch of factor VIII were exposed to a single strain of HIV (Simmonds et al, 1988).

In total 32 haemophiliacs were inadvertently exposed to HIV from this batch between March and May 1984. Eighteen haemophilia patients subsequently developed antibodies to HIV-1 between April and November 1984 while fourteen patients remained seronegative after receiving the contaminated batch of factor VIII. These thirty-two patients form the Edinburgh haemophilia cohort. Individuals exposed to the contaminated batch of factor VIII concentrate who did not develop antibodies to HIV do not show evidence of latent HIV infection in that they do not have any clinical stigmata of HIV, have stable CD 4⁺ counts and are HIV PCR negative (Peutherer et al, 1990). The probability of HIV seroconversion was related to the number of vials of the implicated batch transfused. A batch of factor IX concentrate prepared from the same pool of contaminated plasma was administered to eight patients with haemophilia B and two haemophilia A patients with anti-factor VIII inhibitors. None of these individuals seroconverted.

When compared to other longterm investigations of HIV infection the study of the Edinburgh haemophilia cohort is unique for three important reasons. In the first instance, members of the cohort have been immunologically assessed prior to HIV exposure. Second, the period of exposure to HIV has been precisely defined and finally, the patients are presumed to be infected by the same source; probably a single virus strain.

5.3 Immunological abnormalities relating to HIV infection

Members of the Edinburgh haemophilia cohort have now been assessed clinically and immunologically for a period of eight years.

Patients infected with HIV have shown a progressive decline in mean CD 4⁺ lymphocyte numbers with concomitant increases in plasma β_2 -microglobulin and neopterin. Since 1987 individuals seropositive for HIV antibody have also had raised levels of plasma IgA and increased mean CD 8⁺ T lymphocyte numbers. In contrast, HIV seronegative members of the cohort have remained immunologically stable, although somewhat abnormal (Cuthbert et al, 1990).

A proportion of HIV seronegative haemophilia A patients have unusually high levels of

plasma β_2 -microglobulin, IgG, IgM and IgA in addition to low CD 4⁺ T lymphocyte counts. Immunological abnormalities do not correlate with extent of factor VIII usage or disturbance of liver function tests (Cuthbert et al, 1992).

Three years after initial examination, cell mediated immunity was reassessed in the Edinburgh haemophilia cohort. Cell mediated immunity in patients positive for HIV antibody was found to have declined further. Most patients were completely anergic or responsive to one recall antigen only. The level of cell mediated immunity in HIV seronegative patients has remained constant.

5.4 HIV disease progression

Long term follow-up of the Edinburgh haemophilia cohort has revealed that the rate of progression to clinical AIDS in this group of HIV positive haemophiliacs is relatively rapid. Four years after exposure to HIV 50% of infected patients had progressed to CDC group IV disease (Steel et al, 1988). Ragni and Kingsley (1990) estimate an AIDS incidence of 12%, 28% and 49% after four, six and 7.5 years of infection in a cohort of haemophiliacs in western Pennsylvania. Another report estimated the incidence of AIDS to be 18% six years following seroconversion (Eyster et al, 1987). The calculated ten year cumulative incidence of AIDS was 52% in 63 patients attending the Royal Free Hospital Haemophilia Centre whose date of seroconversion was known (Lee et al, 1990).

In a large study of haemophilia patients in the United Kingdom, Darby et al (1989) report the rate of progression to AIDS in HIV positive individuals depends strongly on age. The cumulative incidence of AIDS five years after seroconversion was found to be 4% among patients aged <25 years at first test positive for HIV, 6% among those aged 25-44 years and 19% among those aged 45 years or over. In contrast, a progression rate of approximately 10% over 6-8 years in Swedish haemophiliacs has been reported by Berntorp et al, (1989), who suggest the use of high purity factor VIII concentrates in the treatment of HIV positive haemophilia patients may be beneficial and postpone progression to AIDS.

There are a number of explanations for the rapid immunological and clinical decline observed in members of the Edinburgh haemophilia cohort. For instance, the cohort

may be infected by a particularly virulent strain of HIV. It is possible that co-infection by other viral pathogens may also contribute to the observed rate of decline in the cohort. For example, cytomegalovirus infection is reported to be associated with more rapid disease progression in HIV positive haemophiliacs (Webster et al, 1989), although this point remains controversial.

It is unlikely that Scottish haemophiliacs suffer from significantly more viral infections than other haemophilia patients, although this has not been formally investigated.

There is good evidence to support a genetic influence on the rate of progression of HIV infection to AIDS in the Edinburgh haemophilia cohort.

In the cohort as a whole a weak association between the HLA haplotype A1 B8 DR3 and an increased risk of seroconversion on exposure to HIV has been noted (Steel et al, 1988). However, in those haemophiliacs who are HIV positive this haplotype is strongly associated with a rapid decline in circulating CD 4⁺ cells and development of HIV related symptoms within four years of infection.

The A1 B8 DR3 haplotype had been associated with the C4A null allele of complement in addition to autoimmune conditions such as systemic lupus erythematosus (Carroll et al, 1987, Dawkins et al, 1983). It has been proposed that A1 B8 DR3 positive individuals are immunologically hyperactive. As HIV replicates preferentially in activated lymphocytes (Margolick and Fauci, 1987), viral replication and dissemination may occur more readily in individuals bearing this haplotype.

The association between A1 B8 DR3 and rapid progression to HIV-related symptoms and AIDS has been confirmed in two non-haemophiliac cohorts (Kaslow et al, 1990, Mallal et al, 1990).

Genetic background may not be the only factor affecting disease progression in the Edinburgh haemophilia cohort. Immunomodulatory effects of locally produced SNBTS factor VIII concentrates may influence the incidence of AIDS in HIV positive haemophiliacs.

5.5 SNBTS intermediate purity factor VIII concentrate

The SNBTS factor VIII concentrate is manufactured at the Protein Fractionation Centre Edinburgh from frozen human plasma (McIntosh et al, 1987). The product is prepared from plasma volumes requiring up to 4 000 donations of plasma using a method based

on extraction from controlled cryoglobulin precipitate (Newman et al, 1971, James and Wickerhauser, 1972). Plasma derived from blood collected locally from volunteer donors is screened for the presence of the hepatitis B surface antigen and since 1985 for antibodies to the human immunodeficiency virus before being used. The concentrate is heated at 80°C for 72 hours in the dried state in order to inactivate blood-borne viruses. The reconstituted product is termed of intermediate purity and contains a mixture of factor VIII, fibrinogen, immunoglobulin, albumin and other plasma proteins in addition to salts and sugars used in formulating the concentrate. Trace contaminants such as metal ions and endotoxins are introduced during the manufacturing process.

6 Factor VIII concentrate and immune modulation

6.1 Effect of factor VIII preparations on lymphocyte activation

Immunomodulatory effects of factor VIII preparations are readily demonstrable *in vitro*. However, it is difficult to compare reports of immunosuppression due to variation in concentrates investigated and specific assay conditions.

(i) Lymphocyte proliferation

North American and Scottish intermediate purity factor VIII concentrates inhibit lymphocyte proliferative responses to non-specific mitogens in a dose-dependent manner *in vitro*. Reductions in PHA- and ConA-induced transformation occur when factor VIII is incubated with normal or haemophiliac peripheral blood lymphocytes (Froebel et al, 1983). Commercial factor VIII concentrates also inhibit lymphocyte transformation following stimulation with the monoclonal antibody OKT₃ (Wang et al, 1985). Purified factor VIII, fibrinogen or fibronectin, at concentrations comparable to those found in commercial factor VIII concentrates cause no inhibition of lymphocyte proliferation. Similarly, the additives glycine and citrate do not consistently inhibit lymphocyte proliferation. Wang et al propose that inhibition of lymphocyte proliferation is mediated by a low molecular weight component of factor VIII preparations. The inhibitory effect is abrogated following concentrate dialysis. It seems unlikely that *in vitro* lymphocyte proliferation is inhibited by factor VIII directly. Neither column-purified factor VIII activity nor antigen causes significant inhibition of PHA-induced lymphocyte stimulation (Daul et al, 1985). Similarly, the

highly purified factor VIII preparation Monoclate is totally devoid of immunosuppressive activity in both human mixed lymphocyte reactions and PHA-induced lymphocyte proliferation assays (Schreiber et al, 1987).

Citrate added to factor VIII preparations as a stabiliser and anticoagulant may well affect proliferative responses to stimulation *in vitro*. McDonald et al (1985) suggest reduced proliferation in response to lectins and the recall antigen purified protein derivative of tuberculin (PPD) are related to citrate concentrations in blood products. Intermediate purity Scottish factor VIII concentrate, stable plasma protein solution and salt-poor albumin, blood products which contain citrate, are all strongly inhibitory to lymphocyte mitogenesis. These products do not inhibit proliferation following dialysis. The authors report that buffer used in the manufacture of Scottish factor VIII and sodium citrate, when present at comparable concentrations, both inhibit lymphocyte proliferation in a dose-dependent fashion. In contrast, human normal immunoglobulin, a citrate-free preparation, has no effect on lymphocyte transformation. Citrate may inhibit lymphocyte proliferation by reducing the availability of calcium and possibly other cations necessary for lymphocyte activation *in vitro*.

Conflicting data identifying two putative inhibitory substances in commercial lyophilized factor VIII preparations are presented by Lederman et al (1986). Gel filtration of factor VIII concentrate revealed two peaks of inhibitory activity of molecular weight $>2 \times 10^6$ Da and approximately 6×10^5 Da. The authors suggest factor VIII itself or a substance bound to or co-migrating with factor VIII inhibits lymphocyte proliferation. In contrast, following gel filtration, fractions of intermediate molecular weight from some factor VIII preparations actually enhanced lymphocyte proliferative responses to PHA. Such fractions did not stimulate lymphocyte cultures in the absence of mitogen. The inhibitory effect of factor VIII transformation appears not to be secondary to an effect on monocyte function (Hay et al, 1990). Factor VIII inhibits lymphocyte proliferation in response to phorbol-myristate-acetate (PMA) in the presence or absence of monocytes. The activation of T cells by PMA is not reliant on the presence of monocytes.

(ii) IL2 production

Factor VIII preparations inhibit the production of interleukin 2 by human lymphocytes

and Jurkat tumour cells following mitogen stimulation (Lederman et al, 1986). As IL2 production by Jurkat cells does not require IL1, it is probable the inhibition of IL2 production induced by factor VIII is not mediated via effects on IL1.

Inhibition of lymphocyte proliferation by factor VIII concentrate is not corrected by the addition of exogenous IL2 (Hay et al, 1990). It is therefore unlikely that factor VIII preparations reduce responsiveness to mitogens and antigens by suppression of IL2 secretion alone.

Thorpe et al (1989) investigated the effects of six factor VIII products used in the United Kingdom on IL2 secretion by human T cells. A range of activities was apparent, varying from total inhibition to no significant effect on IL2 production. No obvious relationship between protein composition and the level of inhibition was found. It is interesting to note, however, that inhibition was observed in the presence of both factor VIII and factor IX preparations, which contain citrate. In contrast, no inhibition occurred in the presence of albumin or immunoglobulin products, which do not contain citrate.

(iii) Activation marker expression

Intermediate purity factor VIII concentrate inhibits the expression of cell surface activation markers on both CD 4⁺ and CD 8⁺ T lymphocytes following stimulation with PHA (Hay et al, 1990). The expression of CD 25, the interleukin 2 receptor, is most profoundly affected but the percentage of peripheral blood lymphocytes expressing the transferrin receptor (CD 71) and HLA-DR positive cells are also reduced. It must be noted that factor VIII was included in proliferation assays at a non-physiological concentration of 4iu/ml. This level of factor VIII activity is not achieved during substitution therapy.

6.2 Effect of factor VIII preparations on monocyte function

In vitro abnormalities in immunological function following exposure to factor VIII are not confined to T lymphocytes.

Factor VIII concentrate preparations have the capacity to modulate monocyte function *in vitro*. Fc receptors expressed on the monocyte plasma membrane are down regulated following a short (1 hour) exposure of normal monocytes to factor VIII concentrates (Eibl et al, 1987). Monocyte effector functions are also impaired in terms of capacity

to kill bacteria and oxygen radical release following stimulation with opsonized zymosan and aggregated IgG.

Fc dependent monocyte phagocytic function is inhibited by factor VIII concentrates, as determined by the ingestion of anti-Rh (D) sensitized O Rh (D) positive erythrocytes (Pasi and Hill, 1990). Monoclonal antibody-purified factor VIII is significantly less inhibitory compared to conventional intermediate purity concentrates. The degree of impaired monocyte function is unrelated to mode of virus inactivation, but decreases as product purity increases.

The contaminant present in factor VIII concentrate responsible for impaired monocyte function may be immune complexes or IgG aggregates (Eibl et al, 1987).

6.3 Effect of factor VIII preparations on NK cell function

NK cell lytic activity of normal peripheral blood lymphocytes against K562 tumour targets is significantly reduced following preincubation with factor VIII (Albin et al, 1986, Lederman et al, 1986).

Published *in vitro* immunomodulatory effects of factor VIII are summarised in Table 1.

The clinical importance of factor VIII or impurities in factor VIII formulations in modulation of immune function both *in vivo* and *in vitro* remains to be clarified. It has implications for the manufacture of all blood products as well as being of importance in deciding upon appropriate factor VIII concentrate therapy for all haemophiliacs.

FACTOR VIII	FACTOR VIII CONCENTRATION (IU/ml)	ASSAY / STIMULUS	IMMUNOMODULATORY EFFECT	REFERENCE
SNBTS Profileate	0.01-1	NK activity PHA con A	No effect on NK activity Dose-dependent inhibition of mitogen responses	Froebelet al, 1983
Swiss Red Cross Behring	0.25-2.5	PHA OKT3	Dose-dependent inhibition	Wang et al, 1985
SNBTS	0.63-5.0	PHA 1µg/ml PWM 1µg/ml con A 20µg/ml PPD 17µg/ml	Dose-dependent inhibition	McDonald et al, 1985
5 commercial concentrates	-	PHA 2-50µg/ml	Dose-dependent inhibition Purified F VIII; no effect	Daul et al, 1985
Koate	-	NK activity	Inhibition	Albin et al, 1986
Hyland Armour Alpha cryoprecipitates	0.0002-1.65	PHA 0.5µg/ml tetanus toxoid IL2 assay NK activity MLR	Dose-dependent inhibition	Lederman et al, 1986

Table 1. Summary of published *in vitro* immunomodulatory effects of various factor VIII preparations.

FACTOR VIII	FACTOR VIII CONCENTRATION (IU/ml)	ASSAY / STIMULUS	IMMUNOMODULATORY EFFECT	REFERENCE
intermediate purity F VIII Monoclate	-	MLR PHA	Inhibition High purity; no effect	Schreiber et al, 1987
Kryobulin M	2-10	CR3 expression FcR expression monocyte function	Reduced	Eibl et al, 1987
Alpha Cutter Baxter BPL SNBTS Armour	1-2.5	PHA OKT3	Range of inhibition of IL2 secretion	Thorpe et al, 1987
Profilate	4	PMA 8ng/ml PHA 2µg/ml	Inhibition of CD25, CD71 and HLA.DR	Hay et al, 1990
Monoclate Factorate Profilate Koate HS Octa V.I. BPL	0.005-0.5	monocyte function	Inhibition; mab-purified product significantly less inhibitory	Pasi and Hill, 1990

Table 1 (continued).

AIMS

The aims of this thesis were as follows;

1. To investigate the nature of effects of various commercial factor VIII preparations on *in vitro* lymphocyte responses to mitogens.
2. To assess quantitative and time course aspects of these effects.
3. To relate effects to the levels of purity of different factor VIII preparations.
4. To derive some information on the possible identity of the contaminant(s) in factor VIII products responsible for the observed immunological effects.
5. To determine whether the observed effects on lymphocyte proliferation might be related to HIV replication *in vitro*.
6. To deduce whether the *in vitro* influence of factor VIII preparations on lymphocyte responses might explain some of the clinical features of disease progression in HIV infected haemophiliacs treated with intermediate purity factor VIII.

The experimental approaches to these aims are summarised below;

1. Standardised lymphocyte proliferation (^3H -thymidine incorporation) assays; titration of mitogens, antigen and stimulator cells. Time course 1-12 days.
2. Compared effects of factor VIII concentrates of differing purity levels in proliferation assays.
3. Related activity *in vitro* to published concentrate composition.
4. Using preparation shown to be active in the above assays (SNBTS Z8),

physical manipulation and fractionation was attempted as follows;

- (i). Filtered and freeze-thawed.
 - (ii). Defibrinated.
 - (iv). Passed through molecular weight cut off filters.
 - (v). Attempted depletion of HLA.DR, factor VIII and fibronectin with specific antibodies.
5. *In vitro* activity of the Z8 and its derived fractions in lymphocyte proliferation assays was assessed simultaneously after each of these stages.
6. Measured effects of factor VIII on cell proliferation and simultaneous virus replication in HIV infected lymphocytes.

MATERIALS AND METHODS

1 Cells

1.1 Peripheral blood mononuclear cells (PBMC)

Peripheral blood mononuclear cells were isolated from venous blood drawn into lithium heparin or from buffy coats (Blood Transfusion Centre, Edinburgh, UK) collected from healthy human blood donations (citrate). Buffy coat samples were defibrinated using sterile orange sticks following the addition of 200 μ l bovine thrombin (Pentex Products, Kankakee, Illinois) reconstituted in 1M calcium chloride. Fresh orange sticks were used to stir the blood gently and remove clots formed until complete defibrination had occurred.

Peripheral blood mononuclear cells were isolated from fresh and defibrinated blood samples by centrifugation over Ficoll/Hypaque (SG 1.077, Pharmacia, Uppsala, Sweden) at 900g for 15 min (50% brake). The interface formed containing mononuclear cells was removed and washed in phosphate buffered saline (PBS).

Residual erythrocytes were removed as required by the addition of 0.17M ammonium chloride for 10 min on ice followed by RPMI 1640 (Gibco, Grand Island, NY) supplemented with 0.03% fresh glutamine and 5% heat inactivated foetal calf serum (FCS). Mononuclear cells were then washed two times in PBS.

All reagents were sterile and sterile handling procedures were followed throughout.

1.2 AET-SRBC

Sheep red blood cells (SRBC) pelleted from defibrinated sheep blood and stored at 4°C in sterile Alsever's solution (Scottish Antibody Production Unit, Carlisle, UK) were centrifuged over Ficoll/Hypaque at 900g for 15 min (50% brake). The interface containing residual sheep mononuclear cells was removed and discarded. One ml SRBC, measured from the packed cell pellet, was washed three times in sterile saline. The final pellet was resuspended in 15 ml 2% aminoethylthiuronium bromide (AET) and incubated at 37°C for 15 min, being shaken every 5 min. AET (Sigma, Poole, Dorset, UK) was freshly prepared, syringe filtered (Acrodisc, Gelman Sciences, Ann Arbor, MI; pore diameter 0.2 μ m) and adjusted to pH 8.0 before use. Following incubation with AET the SRBC were washed four times with sterile saline before being

resuspended in 10 ml RPMI 1640 supplemented with 0.03% fresh glutamine and 40% FCS to make a 10% suspension.

1.3 Separation of T lymphocytes

Adherent cells were removed from peripheral blood mononuclear cell preparations by 60 min incubation in 75 cm² tissue culture flasks at 37°C in 5% CO₂. Cells were incubated in RPMI 1640 with 0.03% fresh glutamine and 10% FCS at a concentration of 1×10^6 - 2×10^6 /ml.

Non-adherent cells were counted and adjusted to 3×10^7 - 4×10^7 /ml in RPMI 1640 with 0.03% fresh glutamine and 40% FCS and mixed with an equal volume of freshly prepared homogeneously resuspended AET-SRBC. One ml aliquots of the suspension were placed in flat bottomed glass Universals and centrifuged at 145g for 5 min before being stored at 4°C for 18 hrs. The majority of the supernatant was removed and the pellet gently resuspended and layered onto a cushion of Ficoll/Hypaque and centrifuged at 900g for 15 min (50% brake). The interface containing non-rosetted, E⁻ mononuclear cells (ie. enriched for B lymphocytes) was removed and discarded. SRBC were removed from the rosetted T lymphocytes in the cell pellet by the addition of 0.17M ammonium chloride for 10 min on ice. The T lymphocytes were then washed once with RPMI 1640 with 0.03% fresh glutamine and 5% FCS, then twice with PBS.

1.4 Lymphoblastoid cell lines

RAB1₂ is an Epstein-Barr virus transformed human B cell line. The RAB1₂ cell line was maintained on RPMI 1640, 0.03% fresh glutamine, 5% FCS in 5% CO₂ at 37°C. The cells were regularly tested for mycoplasma contamination using the Gen-Probe mycoplasma T.C. rapid detection system kit (Gen-Probe Incorporated, San Diego, CA) according to manufacturer's instructions.

The HLA type of RAB1₂ was A2,3 B7/27 DR3,4 W52/W53.

2 Lymphocyte proliferation assays

2.1 Mitogen stimulation

Peripheral blood mononuclear cells or T lymphocyte enriched fractions were cultured at a concentration of 5×10^5 /ml in 200µl aliquots in flat bottomed 96-well Microtest II plates

(Falcon). Cells were cultured in RPMI 1640 supplemented with 0.03% fresh glutamine and 5% FCS (or in some instances 10% autologous human plasma in place of FCS) and incubated at 37°C in 5% CO₂. Cells were stimulated using phytohaemagglutinin (PHA) (Wellcome, Beckenham, Kent, UK, reagent grade) at a final concentration of 1-2 µg/ml or pokeweed mitogen (PWM) (Sigma) at a concentration of 0.5 µg/ml. Assays were performed in the presence of various types of factor VIII, factor IX or control buffer, included at a range of concentrations.

In some instances, peripheral blood mononuclear cells were incubated overnight in culture medium containing SNBTS factor VIII at a final concentration of 2 iu/ml or control buffer diluted to the same extent, before being washed three times and stimulated with 0.5% PHA.

2.2 Antigen stimulation

Peripheral blood mononuclear cells were cultured in RPMI 1640 with 0.03% fresh glutamine and 10% FCS at a concentration of 2.5×10^5 /ml in flat bottomed 96-well plates in 200 µl aliquots. Cells were stimulated with tetanus toxoid (Merieux, Maidenhead, Berkshire, UK) at a final dilution of 1:200.

2.3 One-way mixed lymphocyte reactions

The human B lymphoblastoid cell line RAB1₂ was used in a one-way mixed lymphocyte reaction (MLR) to stimulate peripheral blood mononuclear cells. RAB1₂ cells resuspended in RPMI 1640 supplemented with 5% FCS and 0.03% fresh glutamine were exposed to an X-ray dose of 1 000 rads then washed three times in PBS prior to inclusion in the MLR.

Peripheral blood mononuclear cells and irradiated lymphoblastoid cells were mixed at a ratio of 2.5:1. 150 µl aliquots containing 5×10^5 peripheral blood mononuclear cells and 2×10^5 irradiated lymphoblastoid cells were cultured in 96 v-well plates (Cel-cult). Cells were cultured in the presence of various types and concentrations of factor VIII preparations or control buffer.

In some instances, peripheral blood mononuclear cells and lymphoblastoid cells were preincubated with SNBTS factor VIII at a final concentration of 2 iu/ml for 18 hours before being cultured in a one-way mixed lymphocyte reaction.

2.4 Labelling cells with ^3H -thymidine

Lymphocyte proliferation in response to mitogens, antigens and allogeneic lymphoblastoid cells was measured by incorporation of ^3H -thymidine. Cells were labelled with $1\mu\text{Ci}$ or $0.5\mu\text{Ci}$ ^3H -thymidine (Amersham, Amersham, Bucks, UK) per well in a $50\mu\text{l}$ aliquot of culture medium 18 hours prior to cell harvesting. Replica plates were labelled at 24 hour intervals for up to 12 days.

2.5 Harvesting cells

Cells were harvested onto glass fibre filter paper using a Skatron Multiple Cell-Culture harvester (Skatron, AS, Lier, Norway). Discs were dried and counted in Optiscint "Hi Safe" scintillation fluid (LKB, Loughborough, UK) in plastic vials using a Tri-carb liquid scintillation analyzer. Mean ^3H -thymidine incorporation was calculated for duplicate wells. Alternatively plates were harvested and counted using a Matrix 96 direct beta counter (Packard, Ulgersmaweg, Groningen, The Netherlands). ^3H -thymidine incorporation for replica cultures did not differ by more than 10%.

2.6 Reproducibility of data

Different factor concentrates of varying purity and protein concentration were compared simultaneously in lymphocyte proliferation assays using cells from one blood donor. Samples were performed in duplicate. Assays were performed in general more than one time, with lymphocytes from more than one blood donor. The number of repeat assays performed is summarised in Table 2.

3 Human immunodeficiency virus (HIV) infection

3.1 HIV infection of lymphocytes

Peripheral blood mononuclear cells from a HIV seronegative blood donor were infected with 10^3 logs of HIV strain SF₁₆₂ following a two hour incubation period at 37°C . The infected lymphocytes were then washed three times in RPMI 1640 before being included in proliferation assays.

3.2 HIV inactivation

A range of chemical agents known to inactivate HIV were tested for their effects on

FACTOR CONCENTRATE	PROLIFERATION ASSAY	BLOOD DONORS TESTED	NUMBER OF ASSAYS PERFORMED
SNBTS control buffer	PHA MLR	11 7	16 15
SNBTS Z8-6026	PHA TETANUS PWM	7 3 2	11 3 2
All other batches of SNBTS Z8 [†]	PHA MLR	2(1-7) 3(1-5)	2(1-7) 3(1-6)
BIO PRODUCTS LABORATORY (Elstree) ^{††}	PHA MLR	4(3-5) 1(1-2)	4(3-6) 1(1-2)
SNBTS S8	MLR	2	2
MONOCLATE	PHA	2	2
MONOCLATE-P	PHA MLR	3 5	4 6
PROFILATE	PHA MLR	3 1	3 1
HEMOFIL-M	PHA MLR	2 2	2 2
OCTA-PHARMA V.I. 250	PHA MLR	2 1	2 1
BIO-TRANSFUSION	MLR	2	2
SNBTS FACTOR IX	PHA MLR	2 1	2 1

[†] Average value for 22 batches screened (range).

^{††} Average values for 4 batches screened (range).

TABLE 2

Summary table of assays performed. Table 2 details the number of times each assay was performed giving reproducible results. The number of different blood donors tested per assay is also indicated.

^3H -thymidine incorporation. Replica samples containing culture medium only, 1×10^5 PBMC or 1×10^5 PBMC in 0.1% PHA were cultured in 96-well plates. The cells were labelled for 18 hours with $1 \mu\text{Ci}$ ^3H -thymidine per well, before being incubated for 30 min with either culture medium, 10% TCA, 1% formaldehyde, 2% glutaraldehyde, 20% ethanol or 1% paraformaldehyde (final dilutions). The cells were then harvested and ^3H -thymidine incorporation was assessed by conventional liquid scintillation counting.

3.3 HIV infected lymphocyte proliferation assays

HIV infected or mock infected lymphocytes were stimulated with irradiated lymphoblastoid cells as outlined above. Thirty minutes prior to harvesting paraformaldehyde was added to each well to a final concentration of 1%, in order to inactivate HIV present. Cell cultures were harvested onto glass fibre filters using a Skatron Multiple Cell-Culture Harvester and ^3H -thymidine incorporation measured by liquid scintillation counting.

3.4 Viral quantification

Virus replication was assessed by measuring p24 antigen production in culture supernatants. Cell culture supernatants from duplicate wells were harvested by centrifugation at 24 hour intervals post infection. Supernatants were stored at -20°C until assayed for p24 levels.

HIV p24 antigen was detected using the Coulter HIV p24 Ag enzyme immunoassay (Coulter Corporation, Hialeah, FL), according to the manufacturer's instructions. Briefly, cell culture supernatants to be tested were added to microwells precoated with a murine monoclonal antibody specific for HIV core antigen. If present, antigen complexed to the antibody-coated microwells. The bound antigen was detected by biotinylated antibodies to HIV p24, which reacted with conjugated streptavidin-horseradish peroxidase. Colour developed from the reaction of the peroxidase with hydrogen peroxide in the presence of tetramethylbenzidine substrate. The reaction was terminated by Coulter stopping reagent. The amount of HIV p24 antigen present in the cell culture supernatant was proportional to the intensity of the colour developed. Colour change was measured at a wavelength of 450 nm using a Dynatech MR 700 ELISA plate reader.

4 Cytokine assays

4.1 Conditioned medium

Peripheral blood mononuclear cells were isolated from defibrinated human buffy coat donations and cultured at a concentration of 1×10^6 - 2×10^6 /ml in RPMI 1640 supplemented with 0.03% fresh glutamine, 5% FCS and 1% PHA (Wellcome, reagent grade). Cell cultures were incubated for 24 hours at 37°C in 5% CO₂ before supernatants were harvested by centrifugation. The conditioned medium was then sterile filtered and frozen or stored at 4°C until required.

4.2 Maintaining CTLL-2 cells

Interleukin-2 (IL2) dependent CTLL-2 cells (Gillis and Smith, 1977) obtained from J Simmonds, were maintained on 50% conditioned medium and 50% RPMI 1640 containing 0.03% fresh glutamine and 5% FCS. Cultures were incubated in 5% CO₂ at 37°C in 25cm² tissue culture flasks. Cells were fed every three days and split in a ratio of 1:10.

4.3 Supernatants tested for IL2 activity

2×10^6 peripheral blood mononuclear cells were cultured in 1ml aliquots in 48-well plates (Costar, Cambridge, MA) in RPMI 1640 supplemented with 5% FCS, 1u/ml heparin (Evans Biologicals Ltd, Liverpool, UK) and 0.03% fresh glutamine. The cells were stimulated with 1% PHA (Wellcome, reagent grade) and incubated in the presence or absence of factor VIII at a final dilution of 1iu/ml. Replica wells were harvested by centrifugation at 24 hour intervals and the supernatants stored at -20°C until assayed for IL2 activity. Non-stimulated control wells containing culture medium or factor VIII were also included.

4.4 IL2 bioassay

Supernatants to be assayed for IL2 activity were harvested by centrifugation and stored at -20°C until required. CTLL-2 cells were fed 48 hours prior to inclusion in the IL2 bioassay. CTLL-2 cells were washed twice in PBS, then counted and adjusted to 1×10^5 /ml in RPMI 1640 with 0.03% fresh glutamine and 5% FCS. 100µl of the cell suspension was added to 100µl aliquots of serial doubling dilutions of the test supernatant or standard recombinant IL2 (British Bio-technology Ltd, Oxford, UK) in

culture medium in flat-bottomed 96-well plates (Falcon). Cells were incubated for 24 hours at 37°C in 5% CO₂ before being pulsed with ³H-thymidine. 0.5μCi ³H-thymidine (Amersham) per well was added 5 hours prior to cell harvesting and counting with the Matrix 96 direct beta counter (Packard).

5 Flow cytometry

5.1 Indirect immunofluorescence

Prior to staining for analysis, cell viability was estimated using 0.5% nigrosin dye exclusion. Cells able to exclude the nigrosin dye were scored as viable. To remove debris peripheral blood mononuclear cells were layered onto Ficoll/Hypaque and centrifuged for 15 min at 900g (50% brake). Viable cells at the interface were removed and washed twice with PBS before being stained for flow cytometry. Lymphoblastoid cells of poor viability were removed following centrifugation over a Hypaque cushion (Sigma) at 550g for 15 mins (75% brake). Viable cells at the interface were removed and washed two times with PBS before use. Only cell preparations of greater than 80% viability were stained for analysis.

Cells were washed two times in PBS containing 1% bovine serum albumin and 0.02% azide (PBS.BSA). 5X10⁵ - 1X10⁶ cells were incubated with 50μl or 20μl of the mouse monoclonal antibody of interest for 45 min. Cells were washed twice with PBS.BSA and incubated with 100μl of fluorescein isothiocyanate (FITC) conjugated anti-mouse immunoglobulin (Sigma F2883) at a final dilution of 1:100 for 30 min, in darkness. Cells were washed two times in PBS.BSA before being fixed in 400μl of 1% formaldehyde in PBS. Fixed cells were stored at 4°C in darkness for up to 72 hours before analysis. Cells were analysed using a bench top flow cytometer (FACScan, Becton-Dickenson, Mountain View, CA) equipped with an argon-ion laser emitting light at a fixed wavelength of 488nm. Fluorescence output signals were collected in logarithmic mode. Light scatter signals were collected in linear mode.

5.2 Expression of cell surface activation markers

Peripheral blood mononuclear cells were rosetted overnight with AET-treated sheep red blood cells and the E⁺ T-enriched cells isolated in this manner were incubated in RPMI 1640, 0.03% fresh glutamine and 5% FCS. T-enriched cells were cultured at 37°C in

5% CO₂ at a final concentration of 1×10^6 /ml and stimulated with 0.2% PHA. Cells were cultured in the presence of a variety of factor VIII preparations, present at a range of concentrations, and analysed for the expression of cell surface markers indicative of activation.

Replica cell cultures were therefore labelled with monoclonal antibodies specific for HLA.DR (Becton Dickinson, clone L243), CD 71, the transferrin receptor (Becton Dickinson, clone L01.1) and CD 25, the IL2 receptor (Becton Dickinson, clone 2A3). Control cell cultures were labelled with FITC only and a mouse isotypic control antibody specific for alpha foeto protein, in order to determine the degree of non-specific staining. The expression of CD 44, a cell surface marker whose expression is not normally altered during cell activation, was also investigated using a monoclonal antibody specific for CD 44 (The Binding Site Ltd, Birmingham, UK; clone BU 52) in order to ascertain whether effects of some factor VIII preparations were restricted to the expression of activation markers. CD 44 is a transmembrane protein thought to function as a homing receptor.

The percentage of cells expressing cell surface activation markers in the presence or absence of 2iu/ml SNBTS factor VIII was compared using a paired *t*-test.

5.3 Expression of HLA.DR

The effect of factor VIII concentrate on HLA.DR expression in lymphoblastoid cells was investigated. Irradiated RAB_I₂ cells were incubated in 1ml aliquots at 1×10^6 /ml in RPMI 1640, 5% FCS, 0.03% fresh glutamine, 1u/ml heparin and 2iu/ml SNBTS factor VIII. Control cultures did not contain factor VIII preparations. Lymphoblastoid cells were collected at 24 hour intervals following flotation over a Hypaque cushion (Sigma) and stained as described above for the expression of HLA.DR (Becton Dickinson, clone L243).

5.4 Two colour flow cytometry

Cells responding to irradiated lymphoblastoid B cells were characterized using two colour flow cytometry. 6.6×10^5 peripheral blood mononuclear cells were cultured with 2.6×10^5 irradiated RAB_I₂ lymphoblastoid cells in 2ml aliquots in round-bottomed tubes (Falcon). Cells were incubated in the presence or absence of 2iu/ml SNBTS factor VIII in RPMI 1640 with 5% FCS, 0.03% fresh glutamine and 1u/ml heparin. Cultures were maintained in 5% CO₂ at 37°C for 5 days before being characterized by

two colour flow cytometry.

The mixed cell populations were resuspended and layered onto Ficoll/Hypaque, then spun at 900g for 15 min (95% brake) to remove RAB_I₂ cells and debris. The viable responding cells at the interface were removed and washed two times in PBS.BSA before being stained.

5X10⁵ cells were incubated with monoclonal antibodies specific for CD 71 (Becton Dickinson, clone L01.1), CD 25 (Becton Dickinson, clone 2A3), HLA.DR (Becton Dickinson, clone L243), CD w49 (Serotec, Bicester, Oxon, UK; GI 5), CD 54 (Serotec, 84 HIO), CD 45R0 (UCHL1), CD 3 (Serotec, IOT₃) and CD 19 (3rd Leucocyte typing workshop, BU 12) for 30 min at room temperature. The cells were then washed two times and incubated for 30 min with 100μl of anti-mouse FITC conjugate (Sigma F2883) at a dilution of 1.100, in the dark. Alternatively 5X10⁵ unlabelled cells were incubated for 30 min with a monoclonal antibody specific for CD 29 (Coulter, 4B4) which was directly conjugated with FITC. The cells were washed two times before remaining binding sites on the anti-mouse conjugate were blocked using 50μl of normal mouse serum at a 1.10 dilution. Following a 5 min incubation period 10μl of phycoerythrin labelled monoclonal antibody specific for CD4 (Serotec, Edu-2) or CD8 (Serotec, UCH-T4) were added for a further 30 min incubation period in the dark. The cells were then washed twice in PBS.BSA before being fixed in 400μl PBS with 1% paraformaldehyde. Fixed cells were stored at 4°C protected from the light until analysis.

6 Factor VIII

6.1 Source

A number of commercial and non-commercial factor VIII preparations were included for study. Various batches of Z8 factor VIII, manufactured by the Scottish National Blood Transfusion Service (SNBTS) Protein Fractionation Centre (21 Ellen's Glen Road, Edinburgh) were compared in a number of functional assays. SNBTS Z8 concentrate is manufactured from large volumes of frozen human plasma using a method based on extraction from controlled cryoglobulin precipitate. All plasma donations are screened for evidence of HIV or hepatitis virus infection prior to inclusion in the manufacturing process. In addition SNBTS Z8 is heated to 80 °C for 72 hours in the

dried state. Properties of batches of SNBTS Z8 factor VIII concentrate studied are listed in Appendix I. Buffer, equivalent to the final buffer in which Z8 factor VIII is administered was included for control purposes.

Assays were also performed on 8Y factor VIII manufactured by the Bio Products Laboratory (Plasma Fractionation Laboratory, Churchill Hospital, Oxford), Octapharma V.I.250 (Octapharma, Dusseldorf, Germany), Profilate Heat-Treated (Alpha Therapeutic Corporation, Los Angeles, CA), Bio-transfusion high purity factor VIII concentrate (Bio-transfusion, Lille, France) and monoclonal antibody purified products Monoclate (Armour Pharmaceutical Company, Kankakee, Illinois), Monoclate-P (Armour Pharmaceutical Company) and Hemofil-M (Baxter Healthcare Corporation, Glendale, CA).

BPL 8Y factor VIII is a lyophilised high purity concentrate prepared from large pool fresh frozen plasma. The concentrate is manufactured from volunteer plasma donations collected throughout England and Wales. Plasma included in BPL 8Y factor VIII is screened for antibodies to hepatitis B surface antigen and HIV. After lyophilisation 8Y factor VIII is heat treated for 72 hours at 80 °C.

Bio-transfusion high purity factor VIII concentrate is prepared from plasma by cryoprecipitation followed by ion exchange chromatography. The high purity concentrate produced contains few contaminating proteins and has a factor VIII activity of 42.5-9.5 iu/ml. The concentrate is solvent/detergent treated for virus inactivation. Monoclate factor VIII concentrate is prepared from pooled human plasma collected from paid donors. Factor VIII purification is achieved by immuno-affinity chromatography using mouse monoclonal antibodies raised against human von Willebrand factor antigen (F VIII:R). The final product is stabilised by the addition of human albumin and heat treated for 30 hours at 60 °C. Monoclate-P high purity factor VIII concentrate is prepared in a similar manner but pasteurized, ie. heated to 60 °C for 10 hours in aqueous solution.

Hemofil-M factor VIII is prepared from pooled human plasma by immuno-affinity chromatography using a mouse monoclonal antibody specific for human factor VIII:C. The preparation is further purified by ion exchange chromatography. Human albumin is included in the final product as a protein stabiliser. The preparation is treated with organic solvent and detergent (tri(n-butyl) phosphate and Triton X-100) as a virus inactivation step and lyophilised.

All concentrates were stored and reconstituted according to the manufacturer's

instructions before use *in vitro*. Characteristics of commercial factor VIII preparations are listed in Appendix II.

A newly developed prototype higher purity factor VIII concentrate termed S8 and factor IX concentrate manufactured by the Scottish National Blood Transfusion Service were included in some lymphocyte proliferation assays. The manufacturing process for SNBTS prototype high purity S8 factor VIII concentrate has not been published. SNBTS factor IX concentrate (Defix) is purified from fresh frozen plasma using DEAE cellulose chromatography. Characteristics of SNBTS Z8, SNBTS S8 and SNBTS factor IX concentrates are listed in Appendix III.

6.2 Tissue culture

RPMI 1640 culture medium was supplemented with 1u/ml heparin in addition to 0.03% fresh glutamine and 5% FCS in the presence of factor VIII, following complications of clot formation. Reconstituted factor VIII concentrate was diluted with growth medium and included in assays according to factor VIII activity in iu/ml as stated by the manufacturer. The range of factor VIII activity covered *in vitro* (0.05 - 2iu/ml) was chosen to reflect factor VIII levels in haemophilia patients following factor VIII replacement therapy.

6.3 Cell Viability

The effect of SNBTS factor VIII on peripheral blood mononuclear cell and irradiated lymphoblastoid B cell viability was investigated by 0.5% nigrosin dye exclusion. 1×10^6 cells were incubated in 48-well tissue culture plates (Costar) in replica 1ml aliquots in the presence or absence of 1iu/ml SNBTS factor VIII. Culture medium comprised RPMI 1640, 5% FCS, 0.03% fresh glutamine and 1iu/ml heparin. Cells were incubated at 37°C in 5% CO₂ and viability estimated at 24 hour intervals. 200 cells were counted four times and the mean percentage of nigrosin positive cells was calculated. Cell viability in the presence or absence of SNBTS factor VIII was compared using a paired *t*-test. *P* values of <0.01 were considered significant.

6.4 Defibrinating SNBTS factor VIII

SNBTS factor VIII was reconstituted and defibrinated using sterile orange sticks following the addition of 200µl bovine thrombin (Pentex). Fresh orange sticks were used to gently stir the factor VIII solution until all fibrin clots were removed.

Following defibrination the volume of factor VIII was generally reduced from 20mls to approximately 5mls.

6.5 Physical manipulation of SNBTS factor VIII

Freshly reconstituted and freeze-thawed factor VIII from the same batch were compared in lymphocyte proliferation assays. Similarly, the *in vitro* characteristics of filtered and non-filtered factor VIII were compared. Freshly reconstituted factor VIII was syringe filtered through an Acrodisc membrane of 0.2 μ m pore diameter (Gelman Sciences) before being included in proliferation assays.

A number of ultrafiltration devices of various molecular weight cut off values were used to crudely fractionate factor VIII into a series of broad molecular weight bands.

Defibrinated factor VIII was successively filtered through Centriprep concentrators (Amicon, Danvers, Massachusetts) with molecular weight cut off values of 100,000 Da, 30,000 Da and 10,000 Da according to the manufacturer's instructions. Fractions obtained in this manner were included in lymphocyte proliferation assays *in vitro*.

6.6 Selective protein depletion

In preliminary experiments, specific protein contaminants present in SNBTS factor VIII were targeted for removal by immunomagnetic isolation. Uniform magnetic polystyrene beads were coated with monoclonal antibodies, in an attempt to deplete factor VIII preparations of factor VIII/von Willebrand factor, fibronectin and HLA-DR proteins.

Approximately 4×10^7 Dynabeads M-450 (100 μ l), precoated with goat anti-mouse IgG (Dynal, Oslo, Norway) were resuspended and incubated with 6 μ g of mouse monoclonal antibody specific for factor VIIIc/von Willebrand factor (Chemicon, Temecula, CA; MAB038), fibronectin (Chemicon MAB122) or HLA-DR (Becton Dickinson Clone L243). The beads were rotated for even mixing for 4 hours at 4°C. The Dynabeads were collected with a Dynal Magnetic Particle Concentrator (Dynal MPC 6) and the supernatant discarded. The coated beads were resuspended in PBS containing 0.1% BSA and washed four times for 30 min at 4°C in PBS containing 1% FCS. The beads were collected between washes using the Dynal MPC 6.

Coated beads were incubated with 100 μ l of SNBTS factor VIII (Z8-92490) for 30 min at 4°C using rotation for even mixing. The depleted factor VIII preparations were then removed and stored overnight at room temperature before being assayed in a standard

one-way mixed lymphocyte reaction, as described above.

7 Fibronectin

7.1 One-way mixed lymphocyte reactions

Purified human fibronectin (Chemicon, tissue culture grade) was substituted for factor VIII concentrate in mixed lymphocyte reactions, as described above. Fibronectin was included at concentrations representative of fibronectin content in intermediate purity SNBTS factor VIII preparations (final concentration range 21 μ g/ml-425 μ g/ml).

8 Polyacrylamide gel electrophoresis

8.1 Sample preparation

Factor VIII preparations were reconstituted according to manufacturers' instructions and added directly to 4X sample buffer. Alternatively, concentrate buffer was exchanged for 10mM Tris-HCl prior to the addition of sample buffer. Biogel PD6 (Bio-Rad Laboratories, Richmond, CA) was swollen in 20mM Tris-HCl buffer pH 7.5 and stored at 4°C until required. Micro-columns containing 500 μ l of Biogel PD6 were loaded with 10mM Tris-HCl and centrifuged at 300g for 30 sec at 95% brake two times to allow the column to equilibrate. 100 μ l of factor VIII was then loaded, centrifuged at 300g for 30 sec at 95% brake through the column and collected into a receptacle below. Samples were loaded in 1X SDS sample buffer at a protein concentration of approximately 2mg/ml (4X sample buffer comprised 0.2M Tris-HCl pH 6.5, 20% SDS, 8mM EDTA, 40% glycerol and 0.016% bromophenol blue). 50 μ g and 100 μ g of protein were loaded onto each track for silver and Coomassie Blue staining respectively. Dithiothreitol (DTT) was added to 10mM final concentration for reduced samples. Samples were boiled for 2 min and centrifuged for 4 min in an Eppendorf microcentrifuge prior to sample loading.

8.2 Polyacrylamide gels

Electrophoresis under denaturing conditions was performed using slab gels in the discontinuous buffer system of Laemmli (1970).

Factor VIII samples were electrophoresed through 7% polyacrylamide gels. The separating gel mix comprised 7% acrylamide, 0.187% NN'-methylene bis-acrylamide, 0.375M Tris-HCl pH 8.8 and 0.1% SDS. 30ml of separating gel mix was polymerised following the addition of 48 μ l TEMED and 150 μ l 10% ammonium persulphate.

The stacking gel mix comprised 4.95% acrylamide, 0.132% NN'-methylene bis-acrylamide, 0.125M Tris-HCl pH 6.5, and 0.1% SDS. 10ml of stacking gel mix was polymerised following the addition of 24 μ l TEMED and 75 μ l 10% ammonium persulphate.

Gels were electrophoresed overnight in electrode buffer containing 0.0496M Tris-base, 0.384M glycine and 0.1% SDS.

8.3 Gel staining

(i) Coomassie blue staining

Gels to be stained using Coomassie blue were fixed for 10 min in 10% acetic acid, 20% methanol before being stained for 5 min with 0.125% Coomassie blue R in 50% methanol, 7.5% acetic acid. Gels were destained overnight by soaking in 7% acetic acid, 10% methanol. Stained gels were rinsed and stored in distilled water.

(ii) Silver staining

Gels to be silver stained were fixed at room temperature in a shaking waterbath for 20 min with 20% methanol, 10% acetic acid. The gel was soaked in 50% methanol for 1 hour and rinsed with distilled water for 5 min. This step was repeated two more times before the gel was stained for 15 min at room temperature. The stain solution comprised 0.4% silver nitrate, 0.0756% NaOH, and 0.2072M ammonium hydroxide. The stain was prepared by adding the silver nitrate dropwise to the NaOH and ammonium hydroxide whilst stirring constantly. Following staining, the stain was discarded and the gel rinsed for 5 min with distilled water. The gel was soaked in 0.005% citric acid, 0.0005% formaldehyde (38% stock) developer solution for 5-15 min to allow visualisation of protein bands. The developer was stopped by the addition of 50% methanol, 10% acetic acid. Stained gels were rinsed and stored in distilled water.



RESULTS

1 Effects of factor VIII concentrate on lymphocyte proliferation following stimulation with phytohaemagglutinin

1.1 ^3H -thymidine incorporation

Seventeen of eighteen batches of SNBTS Z8 factor VIII concentrate inhibited lymphocyte proliferation in a dose-dependent manner (Fig 1). When present at a concentration of 2iu/ml, factor VIII reduced ^3H -thymidine incorporation by 85% and delayed the response by 72 hours.

One batch of SNBTS factor VIII (Z8-6026) inhibited lymphocyte proliferation at high concentrations, but enhanced lymphocyte proliferation when present over a lower dose range (Fig 2).

Buffer used in the manufacture of SNBTS factor VIII delayed proliferation when present at a high concentration only (Fig 3).

Four batches of BPL intermediate purity factor VIII concentrate were tested and found to inhibit lymphocyte proliferation in a dose-dependent fashion (Fig 4).

The high purity factor VIII concentrate Profilate inhibited lymphocyte proliferation when present at 2iu/ml, but not at lower concentrations (Fig 5).

High purity Octa-Pharma V.I. 250 did not inhibit lymphocyte proliferation over a range of dilutions tested (Fig 6).

Similarly, monoclonal antibody purified factor VIII preparations Hemofil-M, Monoclata and Monoclata-P had no effect on ^3H -thymidine incorporation (Figs 7, 8, 9).

SNBTS factor IX concentrate inhibited and delayed lymphocyte proliferation in a dose-dependent fashion (Fig10).

SNBTS Z8-81562

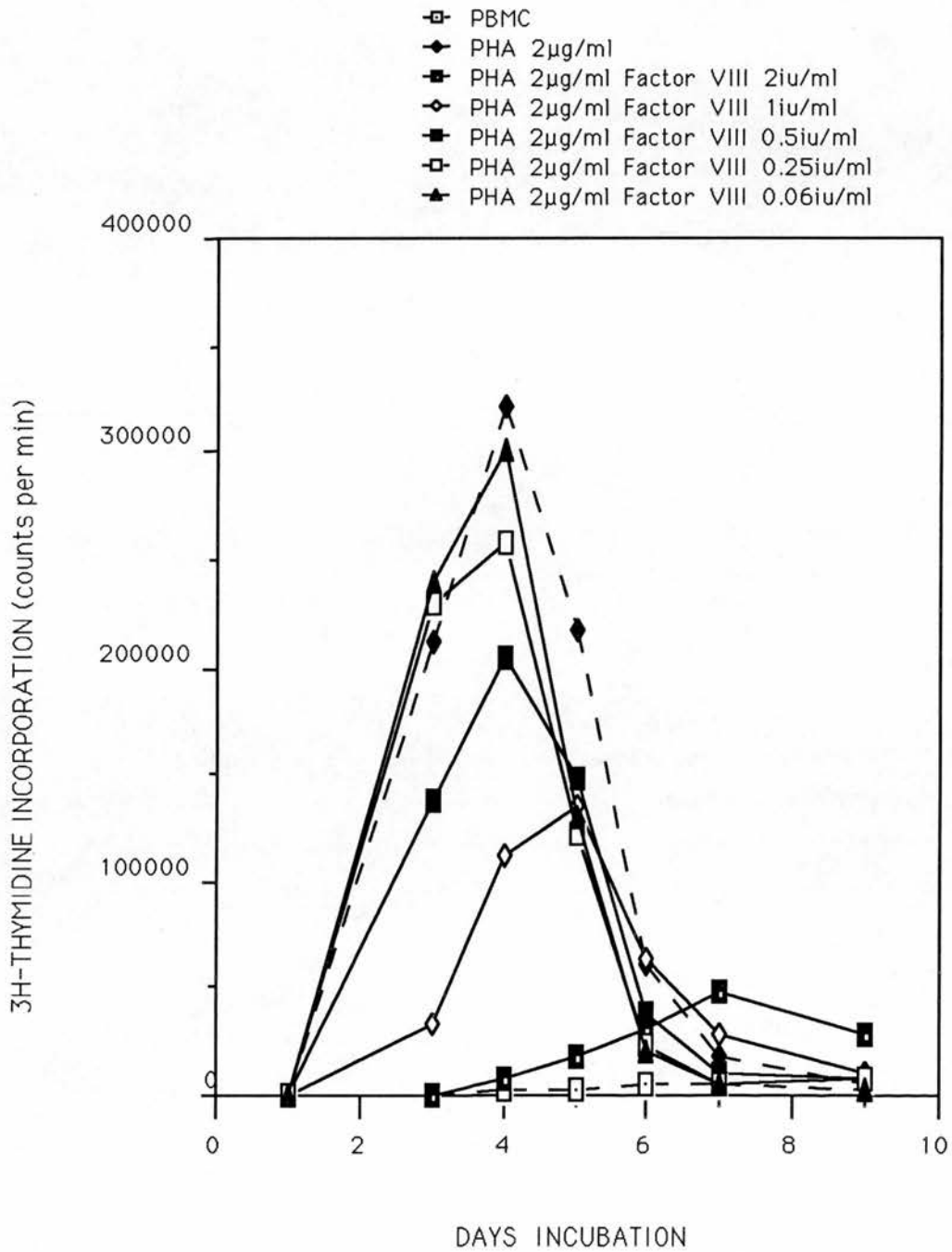


Fig 1
Dose dependent inhibition of lymphocyte proliferation by SNBTS Z8 factor VIII. PBMC were stimulated with PHA at 2 µg/ml. Total protein added per assay ranged from 0.13 mg/ml to 4.18 mg/ml.

SNBTS Z8-6026

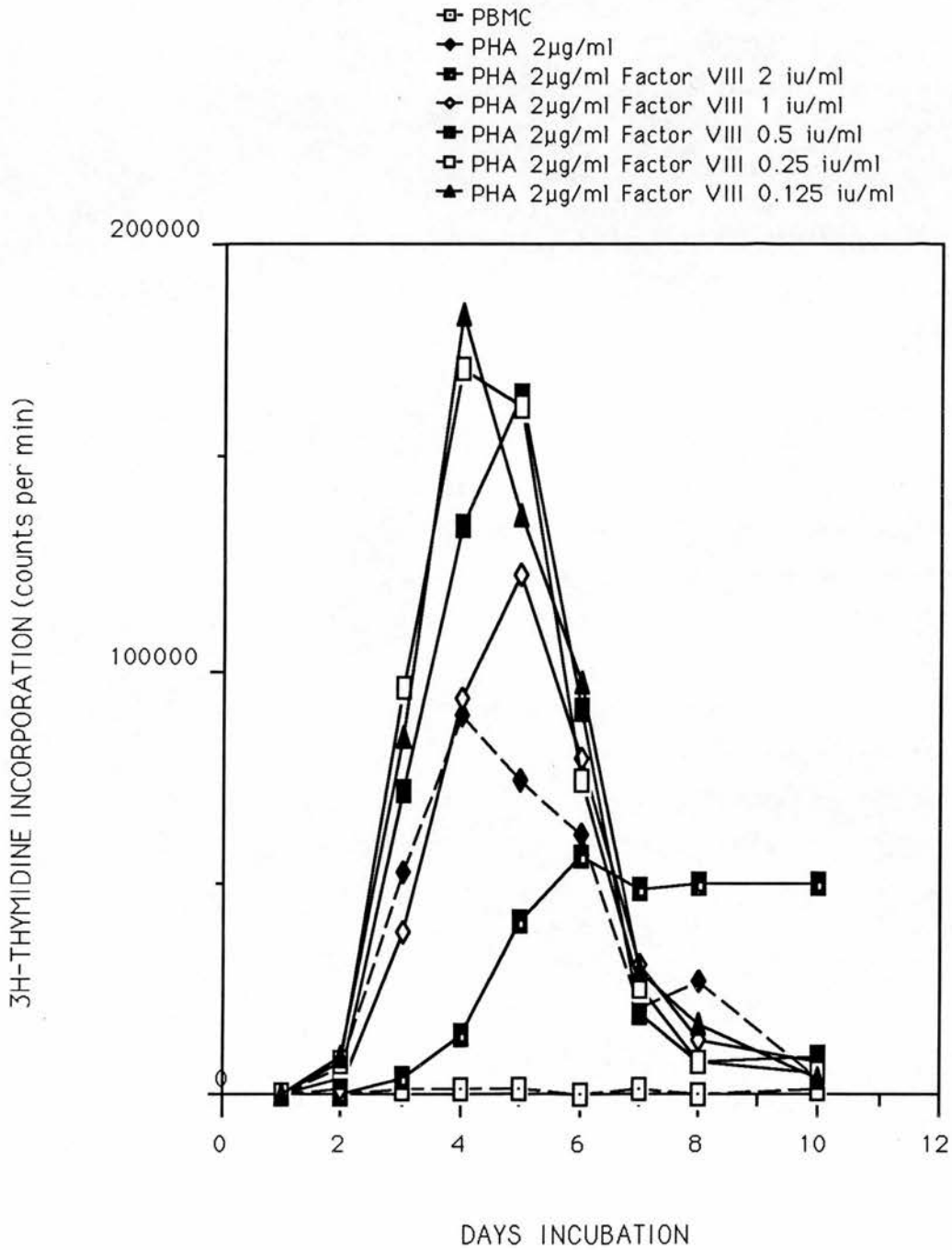


Fig 2
Immunomodulatory effect of SNBTS batch Z8-6026 factor VIII concentrate on lymphocyte proliferation in response to 2 µg/ml PHA. Total protein added per assay ranged from 0.28 mg/ml to 4.41 mg/ml.

SNBTS BUFFER

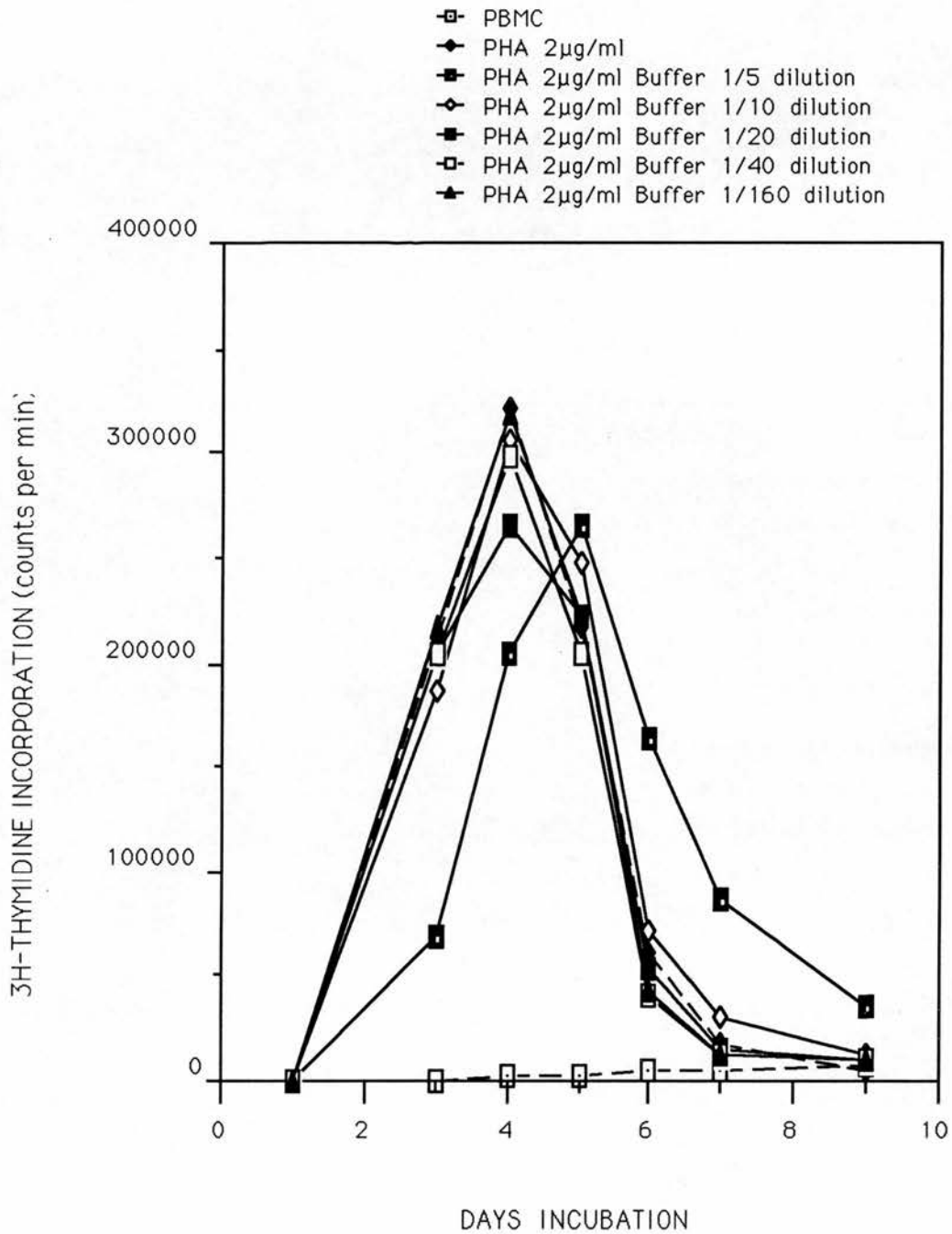


Fig 3

Control buffer used in the manufacture of SNBTS factor VIII concentrate delayed lymphocyte proliferation in response to PHA stimulation when included at a high concentration.

BIO PRODUCTS LABORATORY
8Y-3549

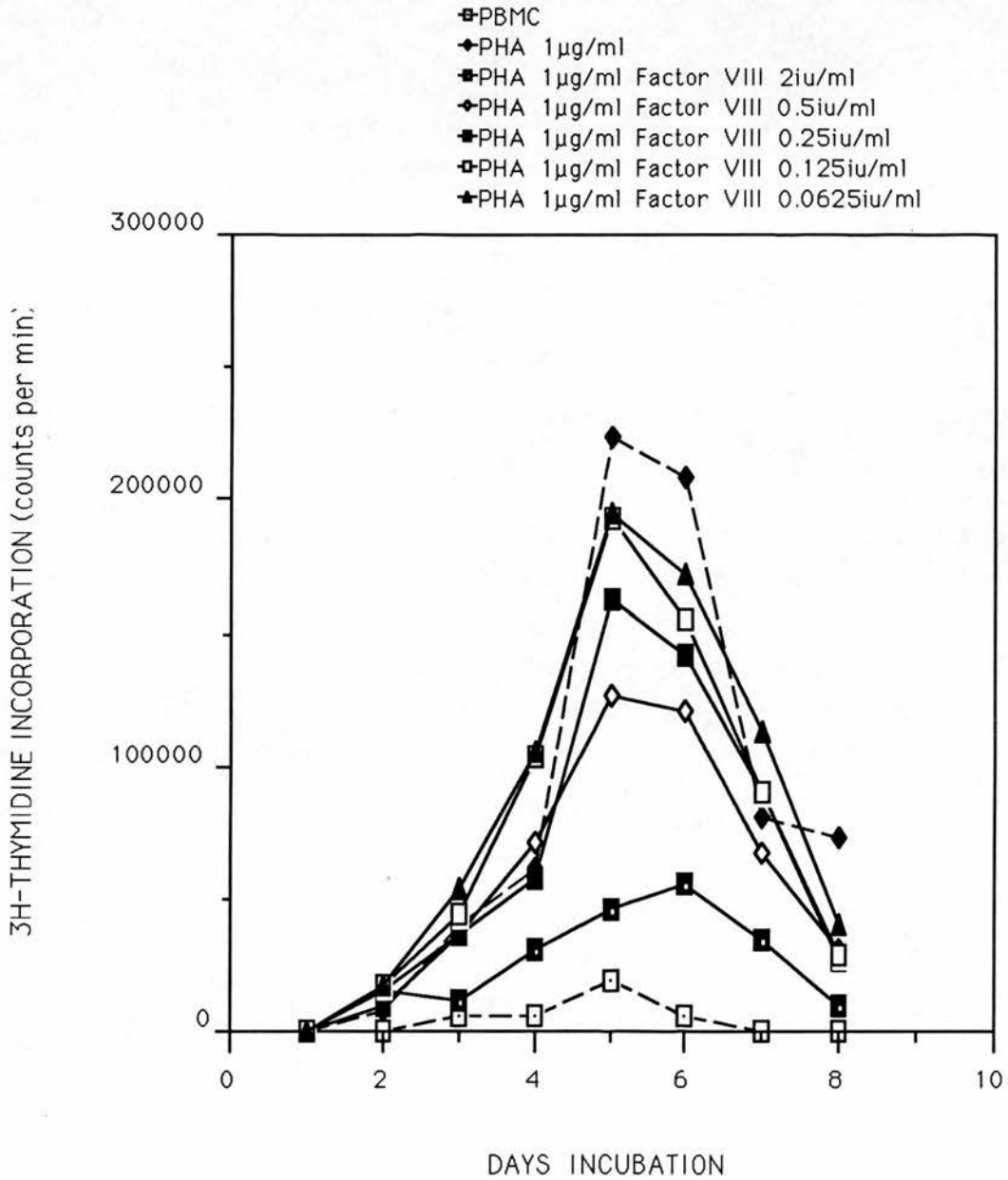


Fig 4

Dose dependent inhibition of lymphocyte proliferation in response to 1 μ g/ml PHA in the presence of Bio Products Laboratory factor VIII. Total protein included per assay ranged from 0.02 mg/ml to 0.51 mg/ml.

PROFILATE

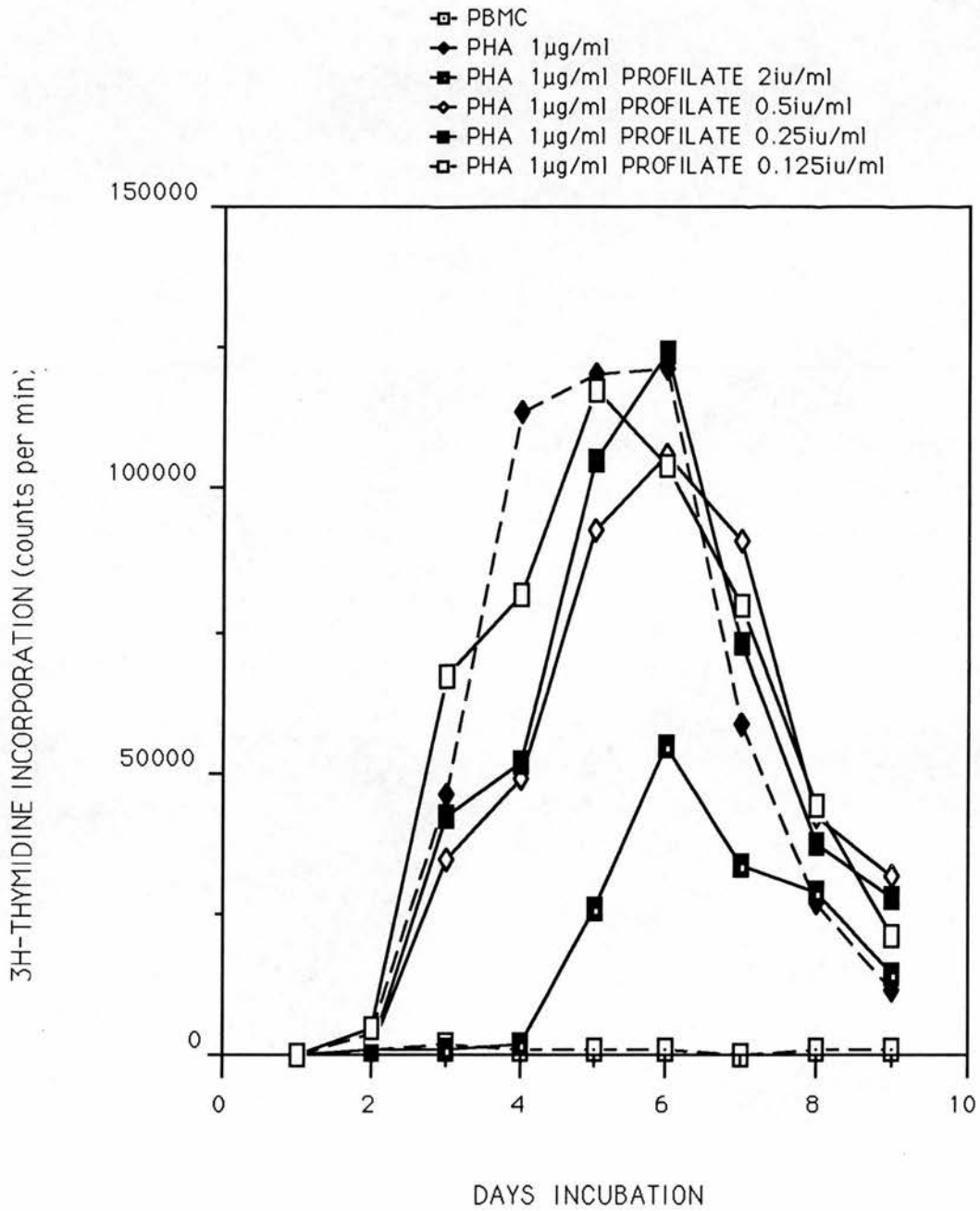


Fig 5
Effect of high purity Profilate factor VIII concentrate on lymphocyte proliferation in response to PHA stimulation. Total protein included per assay ranged from 0.03 mg/ml to 0.45 mg/ml.

OCTA-PHARMA V.I. 250

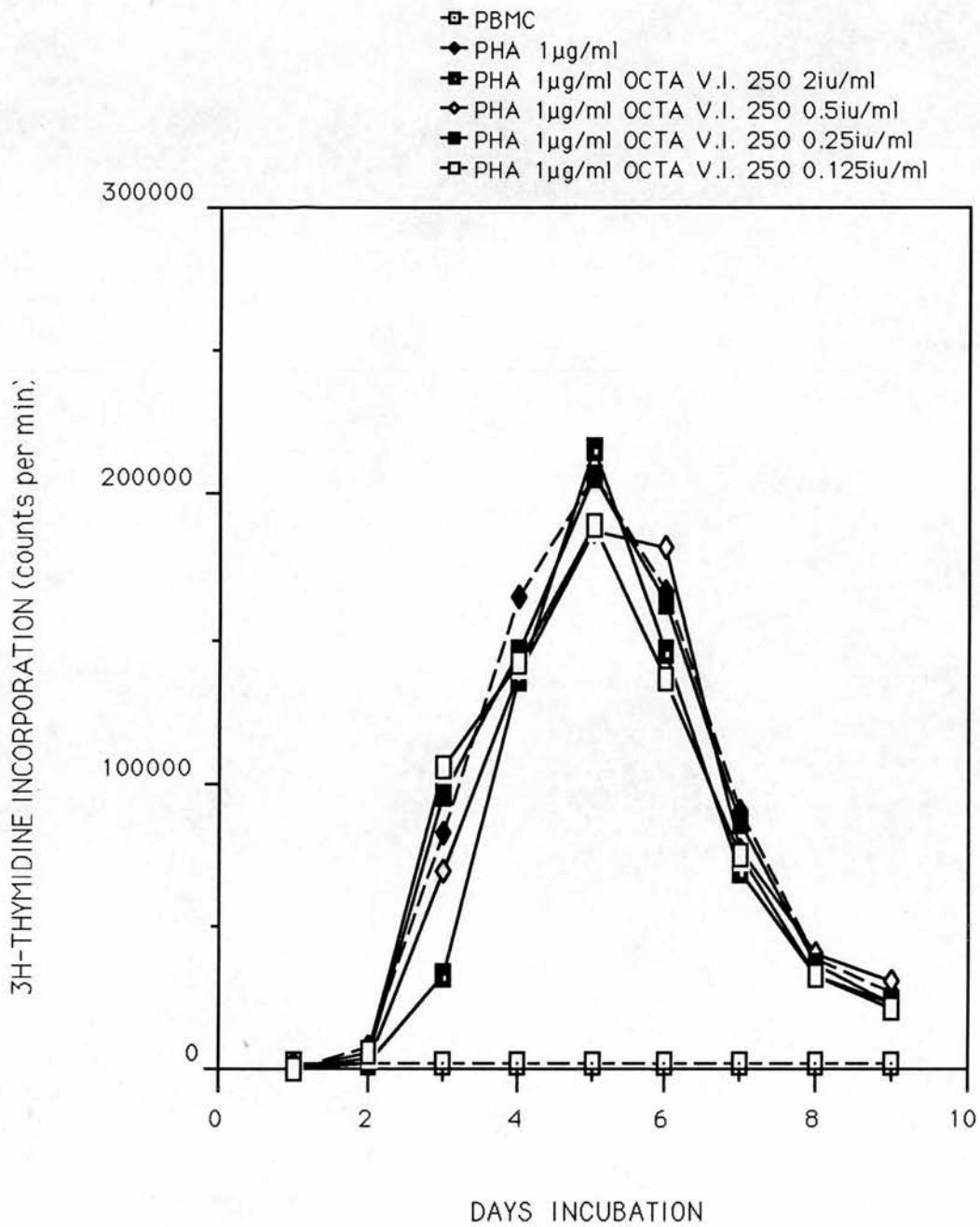


Fig 6

The proliferative response to PHA was not affected by high purity Octa-Pharma V.I. 250 factor VIII over a range of concentrations. Total protein included per assay ranged from 0.03 mg/ml to 0.40 mg/ml.

HEMOFIL-M

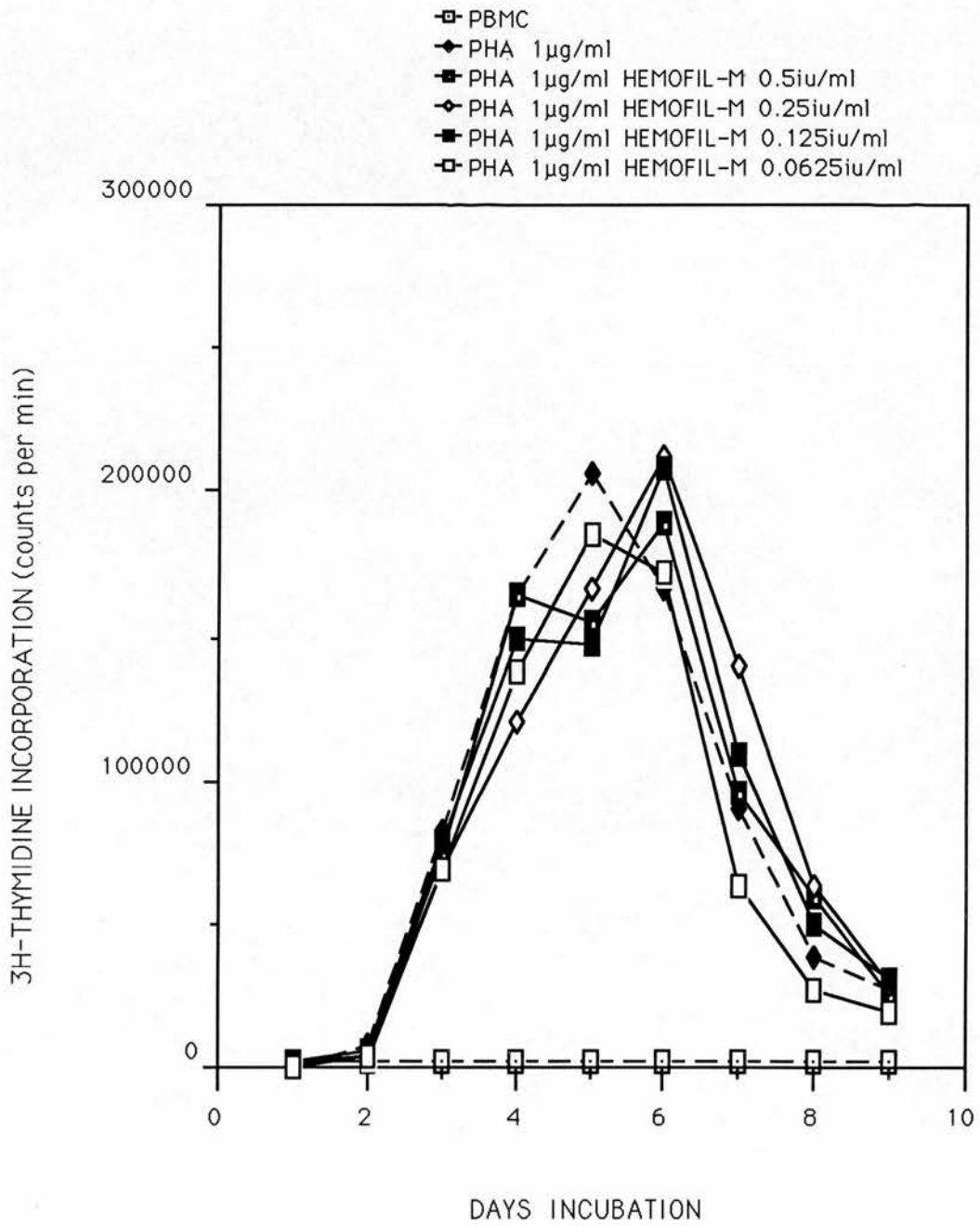


Fig 7
Monoclonal antibody purified Hemofil-M factor VIII concentrate had no effect on lymphocyte proliferation following PHA stimulation.

MONOCLATE D24612

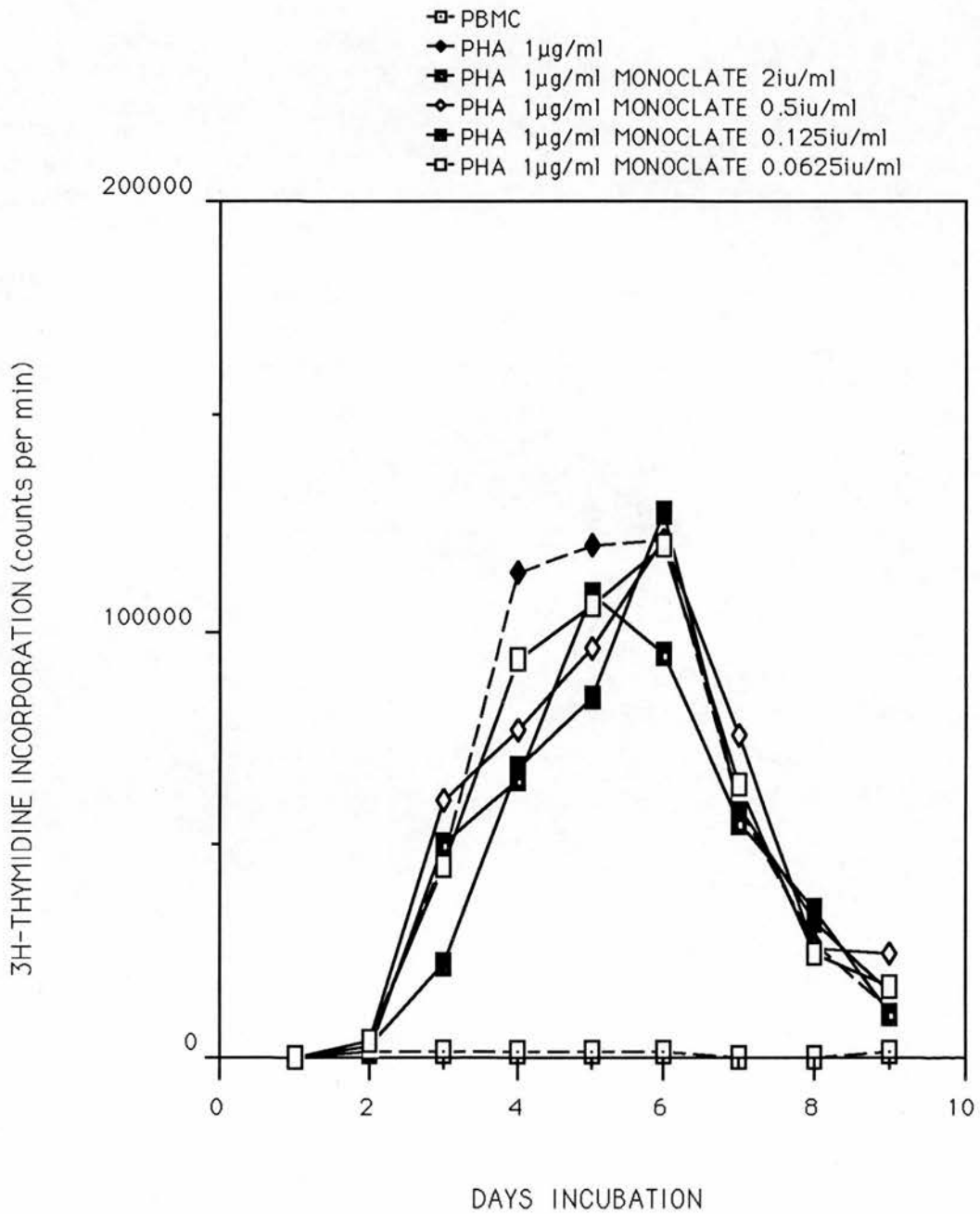


Fig 8
Effect of monoclonal antibody purified Monoclone factor VIII on PHA stimulation of PBMC. Total protein included per assay ranged from 0.004 mg/ml to 0.150 mg/ml.

MONOCLATE-P

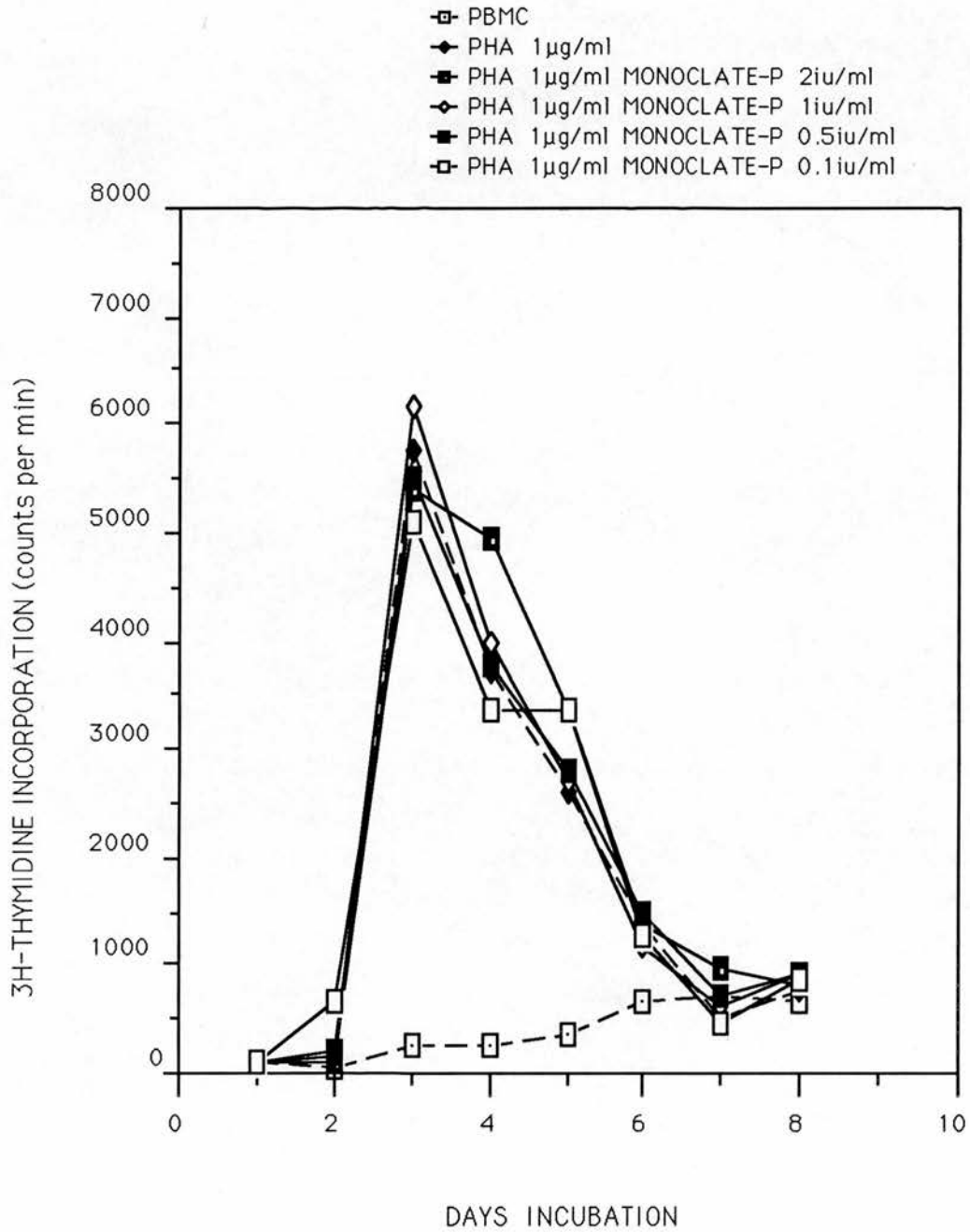


Fig 9

The proliferative response to PHA was not affected by pasteurized monoclonal antibody purified Monoclote-P factor VIII. Total protein included per assay ranged from 0.007 mg/ml to 0.150 mg/ml.

SNBTS FACTOR IX

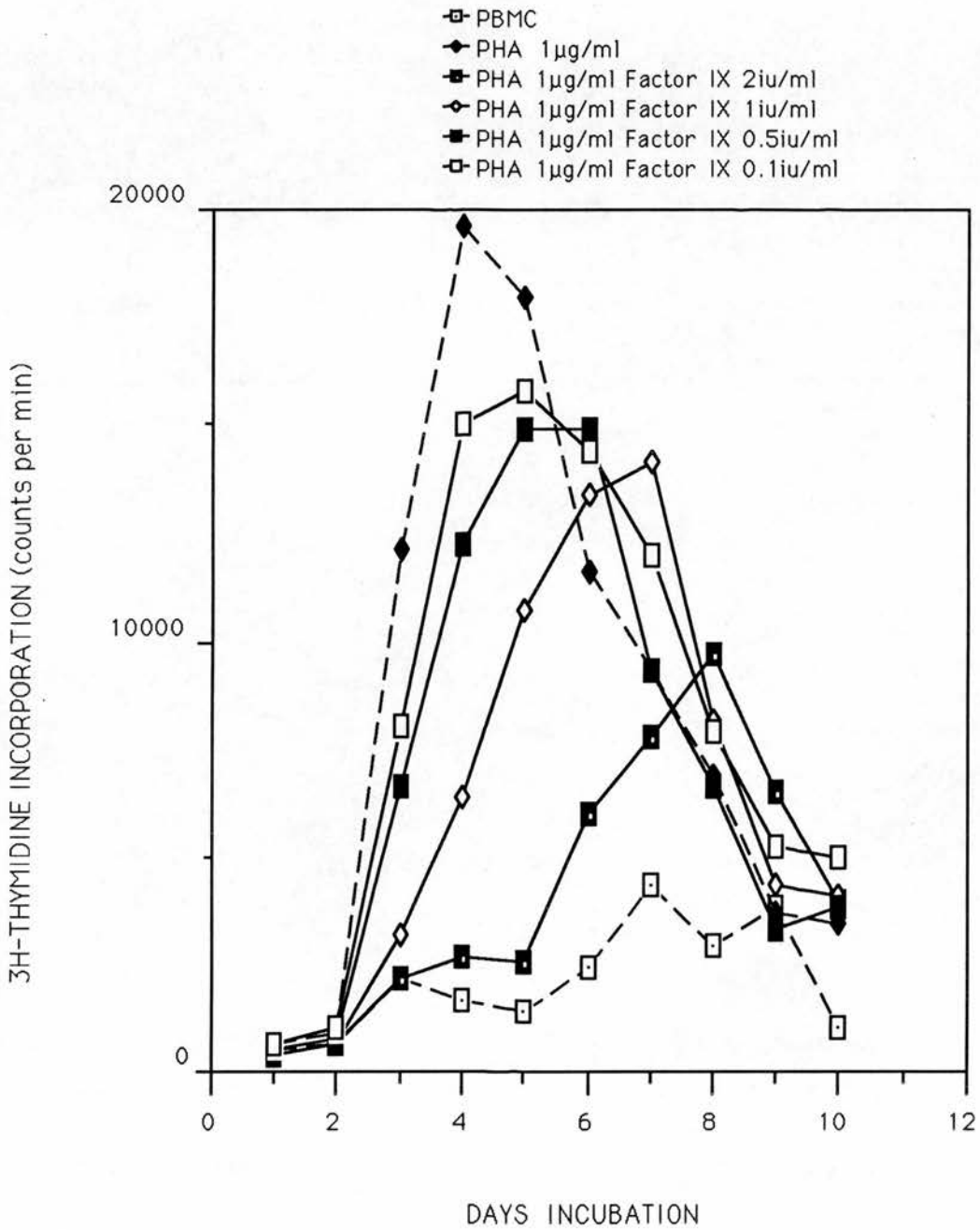


Fig 10

SNBTS factor IX inhibited and delayed PBMC proliferation in response to PHA in a dose dependent fashion. Total protein included per assay ranged from 0.04 mg/ml to 0.88 mg/ml.

1.2 Cell surface activation marker expression

I have confirmed the observation by Hay et al (1990) that factor VIII concentrate altered lymphocyte activation marker expression *in vitro*.

Factor VIII concentrate or control buffer were incubated with mononuclear cell populations enriched for T cells and stimulated with PHA.

Flow cytometric analysis showed that both SNBTS factor VIII concentrate and buffer caused a reduction in the percentage of cells expressing CD25 and CD71 in a dose-related fashion. Neither concentrate nor buffer altered the expression of CD44 or HLA-DR (Figs 11, 12).

The expression of CD25 in the presence of 2iu/ml factor VIII and CD71 in the presence of control buffer at a dilution of 1/5.75 was significantly reduced ($P < 0.05$).

All other differences failed to reach significance.

The percentage of cells analysed staining positive for CD5, CD14 and CD19 were 97%, 0.4% and 1.5% respectively.

Incubation of Monoclone-P with T-enriched cells stimulated with PHA had no effect on activation marker expression (Fig 13).

In a comparative study, SNBTS S8 prototype higher purity factor VIII concentrate was found to reduce the percentage of cells expressing CD25 and CD71 to the same extent as SNBTS intermediate purity factor VIII. The percentage of cells expressing activation markers was reduced to a lesser extent by control buffer (Figs 14, 15, 16).

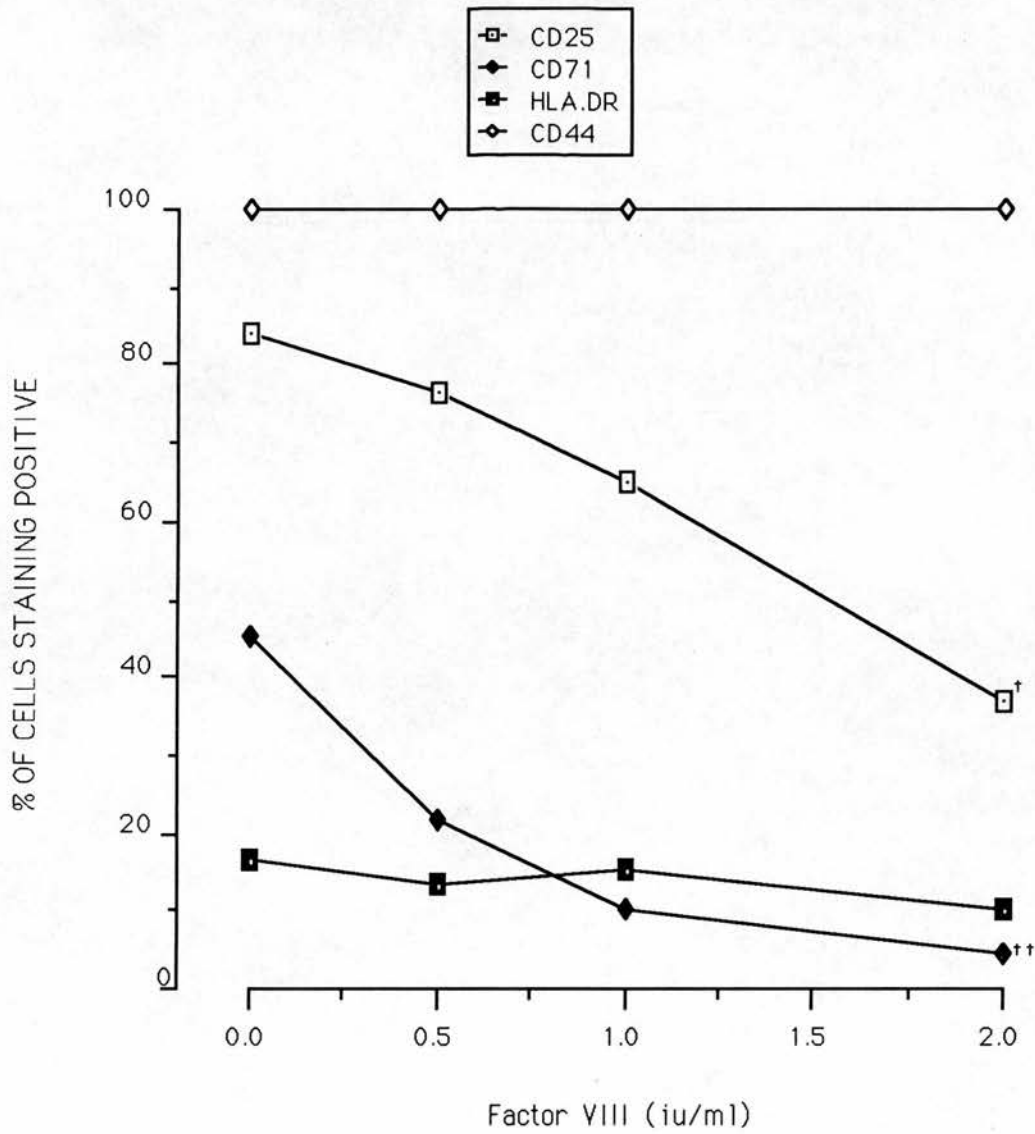
The expression of CD25 in the presence of 2iu/ml SNBTS Z8, 2iu/ml SNBTS S8 or 1/5.75 control buffer was significantly reduced ($P < 0.05$). All other differences failed to reach significance.

1.3 IL2 production

I have confirmed the observation by Thorpe et al (1989) that factor VIII concentrate altered IL2 secretion by peripheral blood mononuclear cells *in vitro*.

Levels of IL2 were assayed by measuring the proliferative response of IL2 dependent CTLL-2 cells using ^3H -thymidine incorporation. IL2 levels were assayed at 0, 24 and

SNBTS Z8-92340

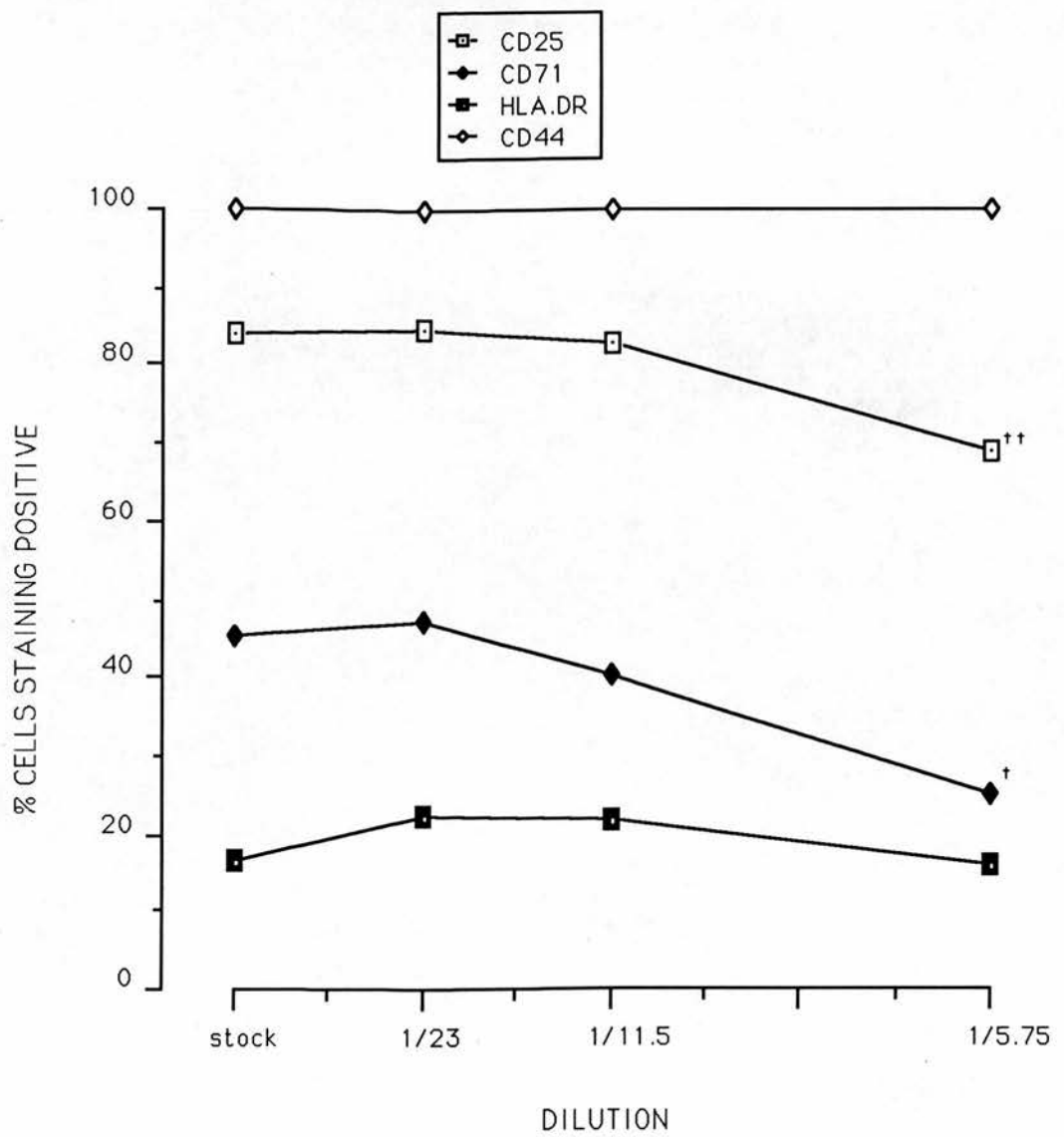


[†] $P < 0.05$

^{††} $P < 0.10$

Fig 11
 Percentage of cells staining positive for activation markers CD 25, CD 71 and HLA.DR following PHA stimulation in the presence of SNBTS Z8 factor VIII. Total protein included per assay ranged from 0 mg/ml to 4.5 mg/ml.

SNBTS BUFFER



[†] $P < 0.05$

^{††} $P < 0.10$

Fig 12

The effect of control buffer on activation marker expression following stimulation with the mitogen PHA.

MONOCLATE-P

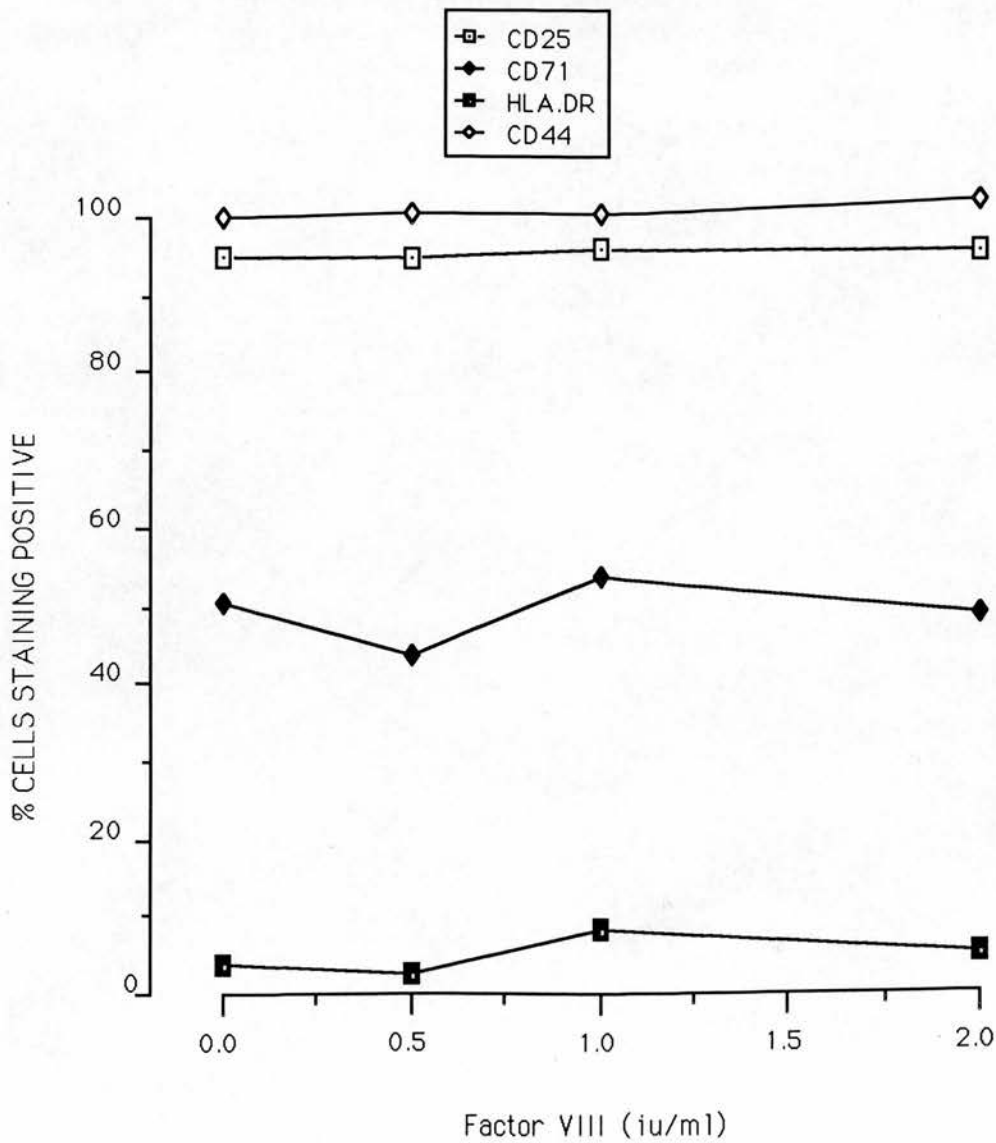


Fig 13

The expression of activation markers CD 25, CD 71 and HLA.DR following PHA stimulation was not affected by the presence of Monoclote-P factor VIII concentrate. Total protein included per assay ranged from 0 mg/ml to 0.15 mg/ml.

SNBTS Z8-92340

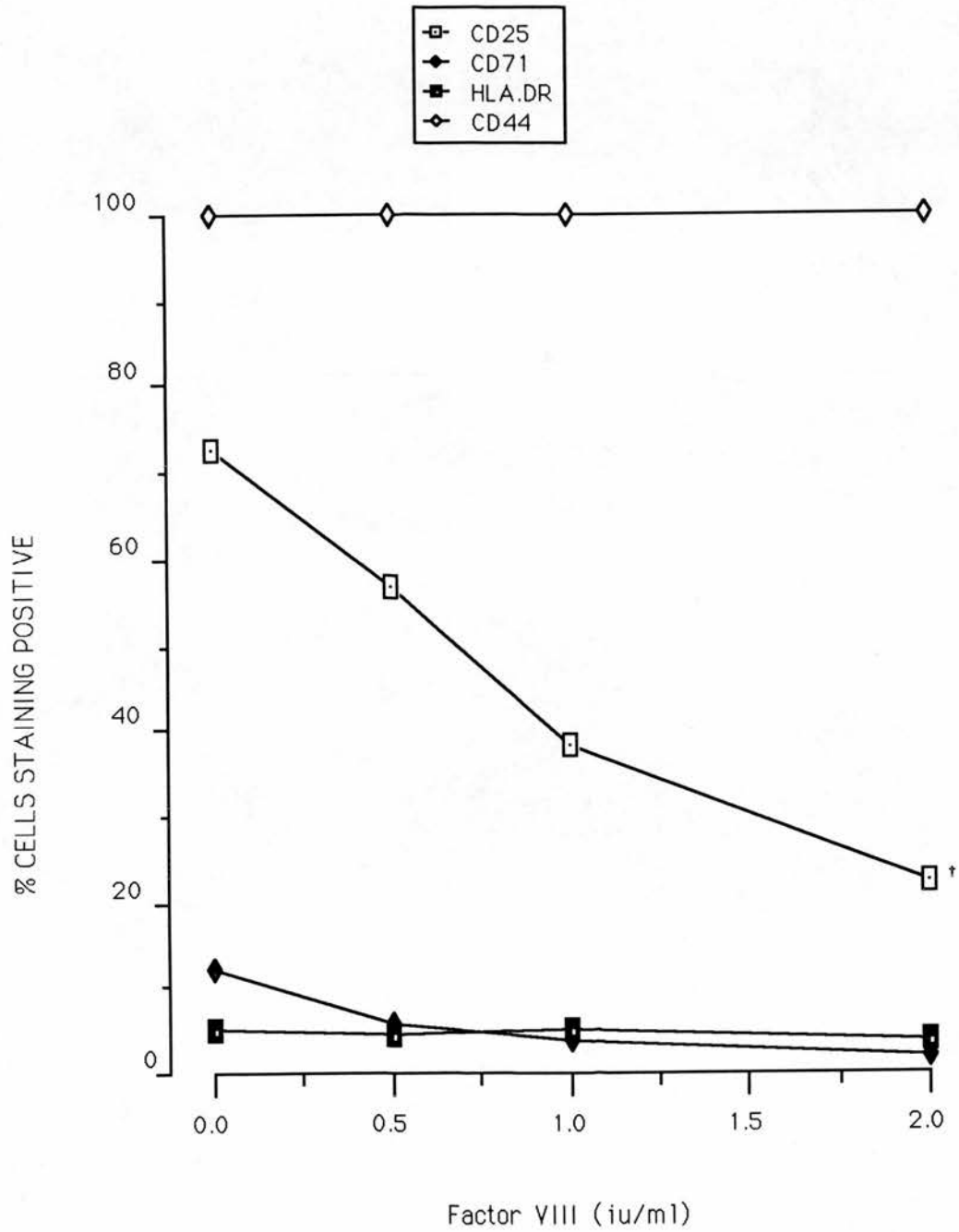


Fig 14
 Effect of SNBTS Z8 factor VIII on activation marker expression following PHA stimulation. Total protein included per assay ranged from 0 mg/ml to 4.5 mg/ml.

SNBTS S8-0006

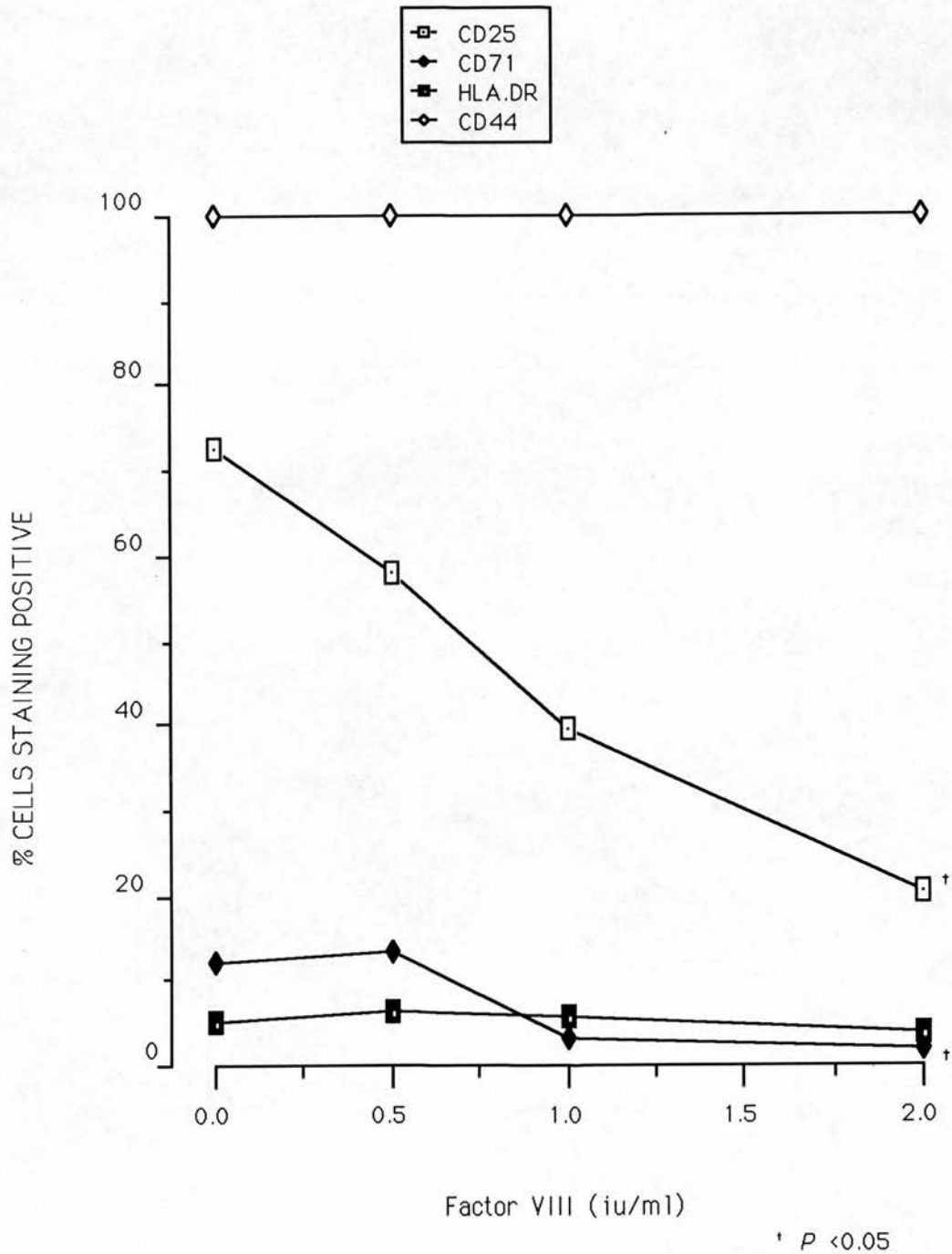
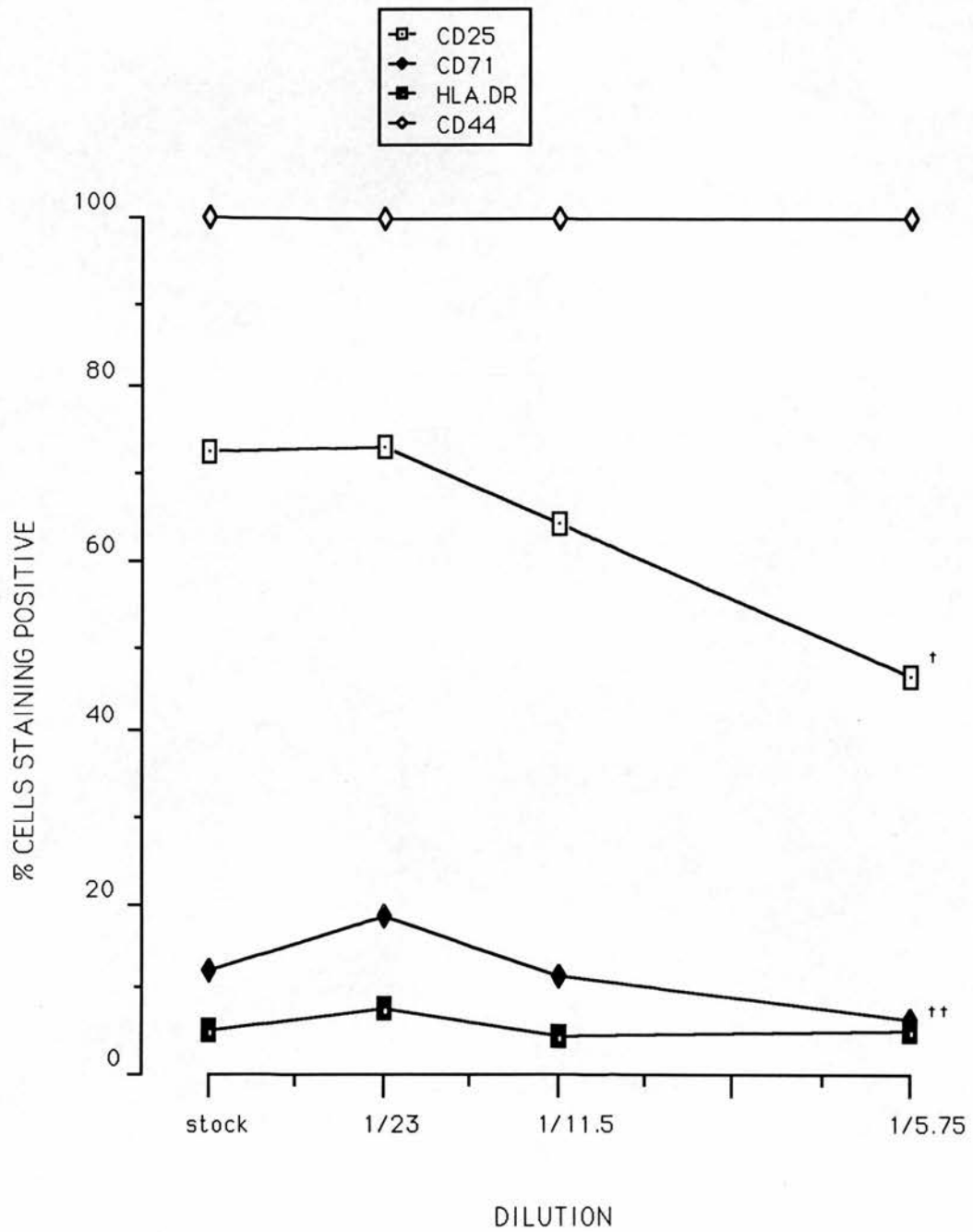


Fig 15
 Effect of prototype high purity SNBTS S8 factor VIII concentrate on activation marker expression following PHA stimulation. Total protein included per assay ranged from 0 mg/ml to 1.5 mg/ml.

SNBTS BUFFER



[†] $P < 0.05$

^{††} $P < 0.10$

Fig 16
Effect of SNBTS control buffer on activation marker expression following PHA stimulation.

48 hours post stimulation in the presence or absence of 1iu/ml SNBTS factor VIII. Neither factor VIII nor PHA affected ^3H -thymidine incorporation in response to recombinant IL2. Peripheral blood mononuclear cells stimulated with PHA produced lower levels of IL2 in the presence of factor VIII concentrate (Fig 17). At 24 hours post stimulation IL2 secretion was reduced approximately two fold in the presence of 1iu/ml SNBTS factor VIII.

Values were converted to a percentage of the maximum counts per minute obtained and IL2 values calculated (Fig 18, Table 3). Points on the dilution axis corresponding to 50% of the maximum counts per minute are designated 1 unit of IL2 activity.

1.4 Preincubation of SNBTS Z8 factor VIII with peripheral blood mononuclear cells

Significant inhibition of lymphocyte proliferation following PHA stimulation only occurred if factor VIII concentrate was present throughout the culture period.

Peripheral blood mononuclear cells incubated overnight with 2iu/ml SNBTS factor VIII responded normally to PHA (Fig 19).

1.5 Filtered SNBTS factor VIII concentrate

In a comparative series, factor VIII filtered through a membrane (0.2 μm pore diameter) behaved identically to fresh concentrate from the same batch. 100% and 50% inhibition was observed at concentrations of 5iu/ml (1/2.4 dilution) and 2iu/ml (1/6 dilution) respectively. Control buffer partially reduced and delayed the proliferation response when present at a high concentration (Fig 20, 21, 22).

2 Effect of SNBTS factor VIII batch Z8-6026 on lymphocyte proliferation following stimulation with tetanus toxoid

SNBTS factor VIII batch Z8-6026 inhibited the proliferation response to the recall antigen tetanus toxoid completely when present at 2iu/ml. Proliferation was partially reduced when the concentrate was included at a concentration of 1iu/ml. In contrast, the response to tetanus toxoid was markedly enhanced by this particular batch of SNBTS factor VIII when present over a lower dose range (Fig 23).

CTLL-2 assay
 SNBTS Z8-92490
 PHA STIMULATION

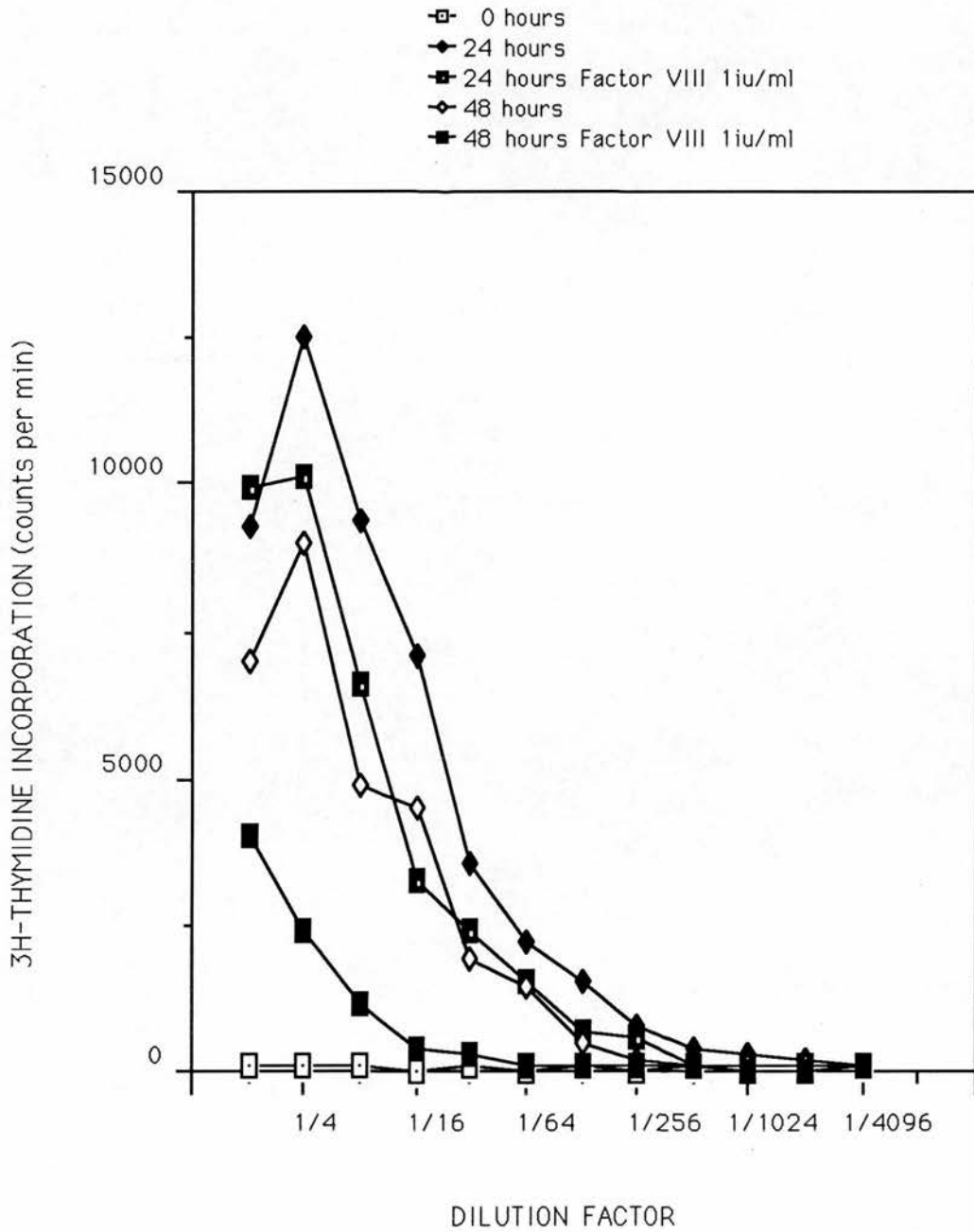


Fig 17
 Proliferative response of IL2 dependent CTLL-2 cells to culture supernatants harvested from PBMC stimulated with PHA in the presence or absence of SNBTS Z8 factor VIII.

CTLL-2 ASSAY
 SNBTS Z8-92490
 PHA STIMULATION

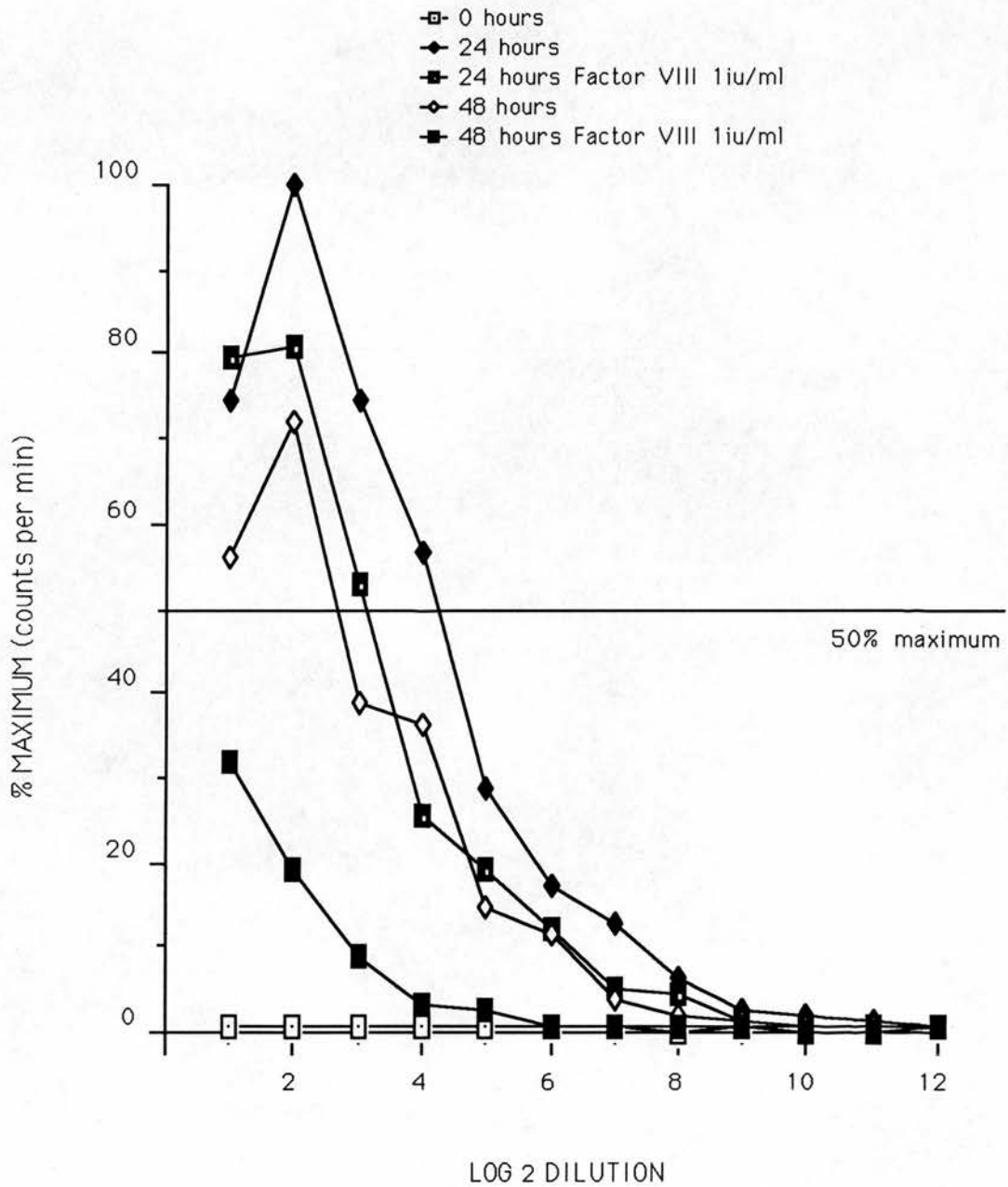


Fig 18

Values obtained in CTLL-2 bioassay converted to percentage of maximum counts per minute. Points on the dilution axis corresponding to 50% of the maximum counts per minute are designated 1 unit of IL2 activity.

CTLL-2 ASSAY
 SNBTS Z8-92490 (1iu/ml)
 PHA STIMULATION

	1/2 maximal value	IL2 (U/ml)
0 hours	-	0
24 hours	24.4	21.11
24 hours F VIII	23.3	9.85
48 hours	22.8	6.96
48 hours F VIII	<21.1	<2.14

Table 3
 IL2 production following PHA stimulation in the presence or absence of 1 iu/ml SNBTS Z8 factor VIII concentrate. IL2 production was assayed at 24 hours and 48 hours post stimulation. Values for ³H-thymidine incorporation were converted to a percentage of the maximum counts per minute obtained. Points on the dilution axis corresponding to 50% of the maximum counts per minute are designated 1 unit of IL2 activity.

PREINCUBATION OF SNBTS FACTOR VIII (Z8-92450) WITH
PERIPHERAL BLOOD MONONUCLEAR CELLS.

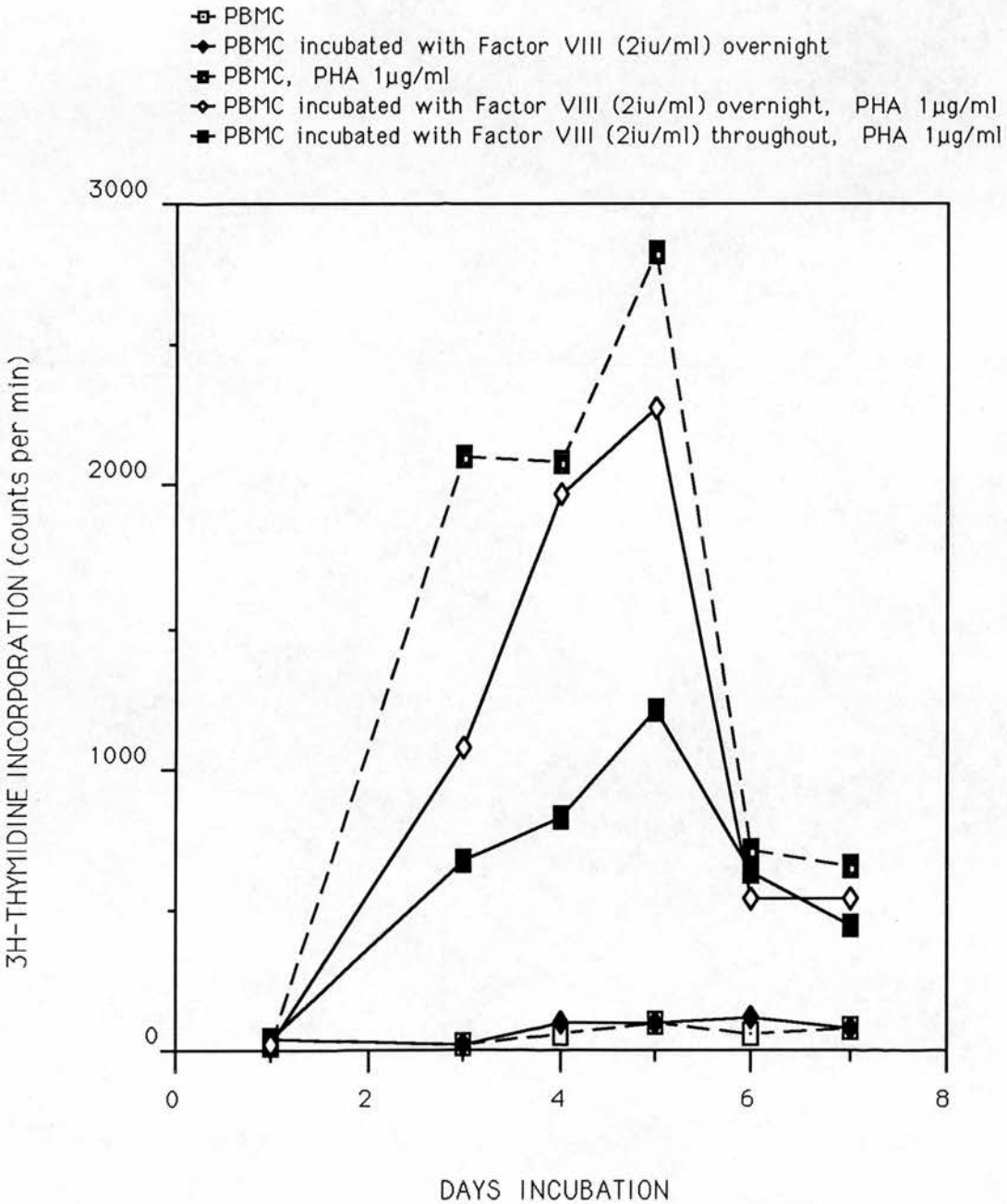


Fig 19
Effect of preincubation of PBMC with factor VIII concentrate prior to activation.
Inhibition of lymphocyte proliferation in response to PHA stimulation was
dependent on the continued presence of factor VIII concentrate.

SNBTS Z8-02560
FRESH

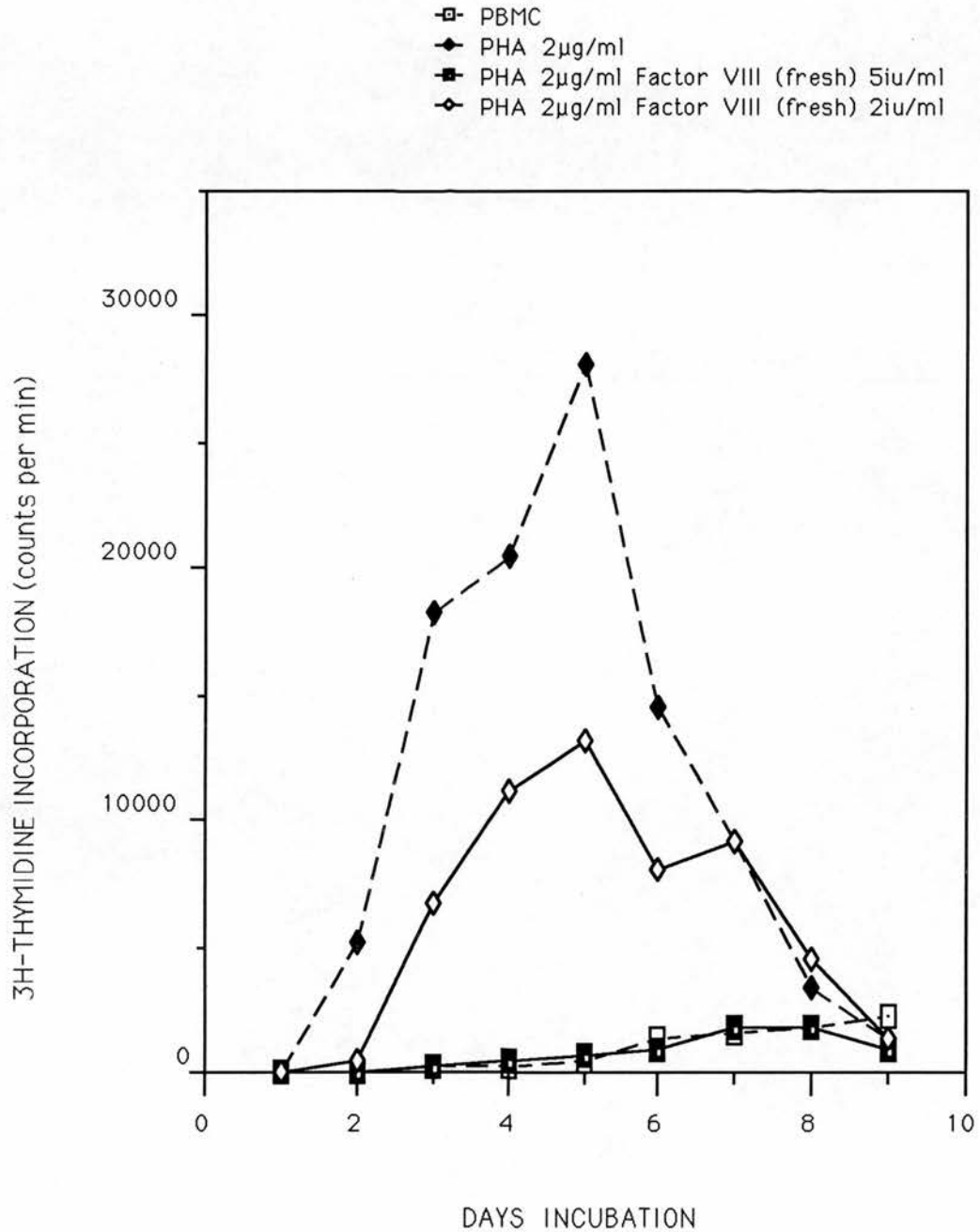


Fig 20
Effect of freshly reconstituted SNBTS Z8 factor VIII on PHA stimulation at two concentrations.

**SNBTS Z8-02560
FILTERED**

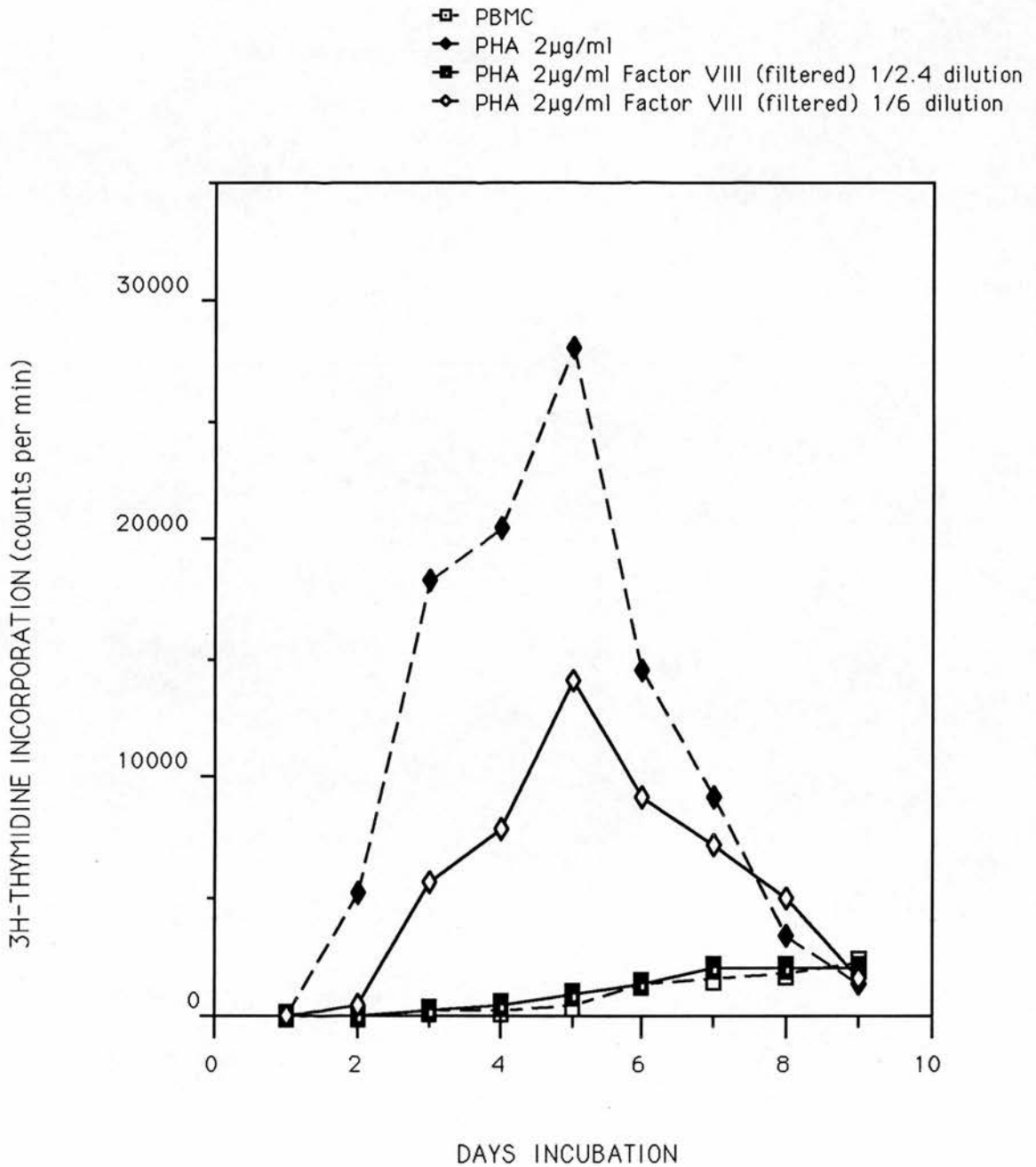


Fig 21

Effect of SNBTS factor VIII filtered through a 0.2 μ m pore sized membrane on PHA stimulation, included at comparable dilutions to those in Fig 20.

SNBTS BUFFER

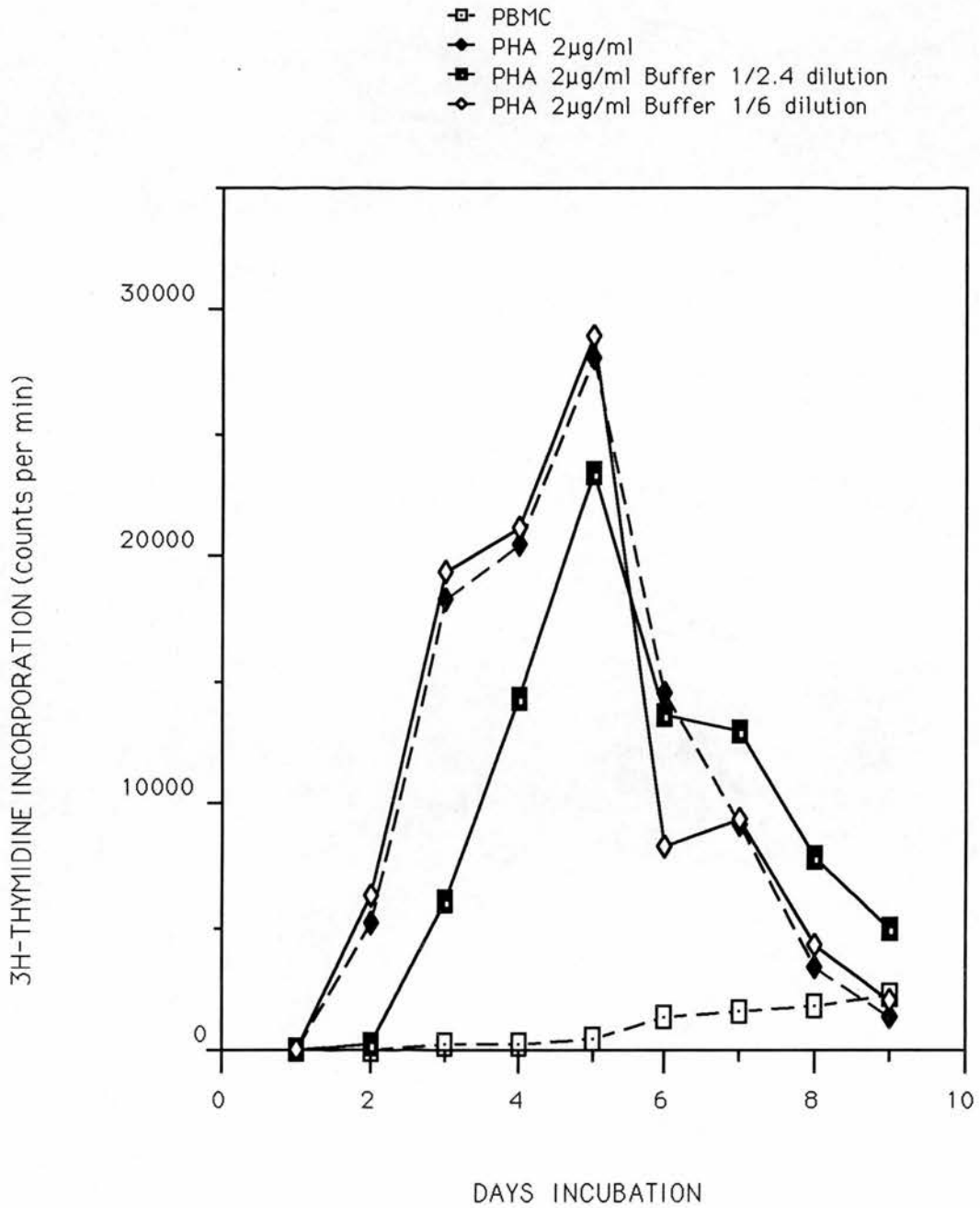


Fig 22

Effect of SNBTS control buffer on lymphocyte proliferation in response to PHA at comparable dilutions to those in Fig 20.

SNBTS Z8-6026

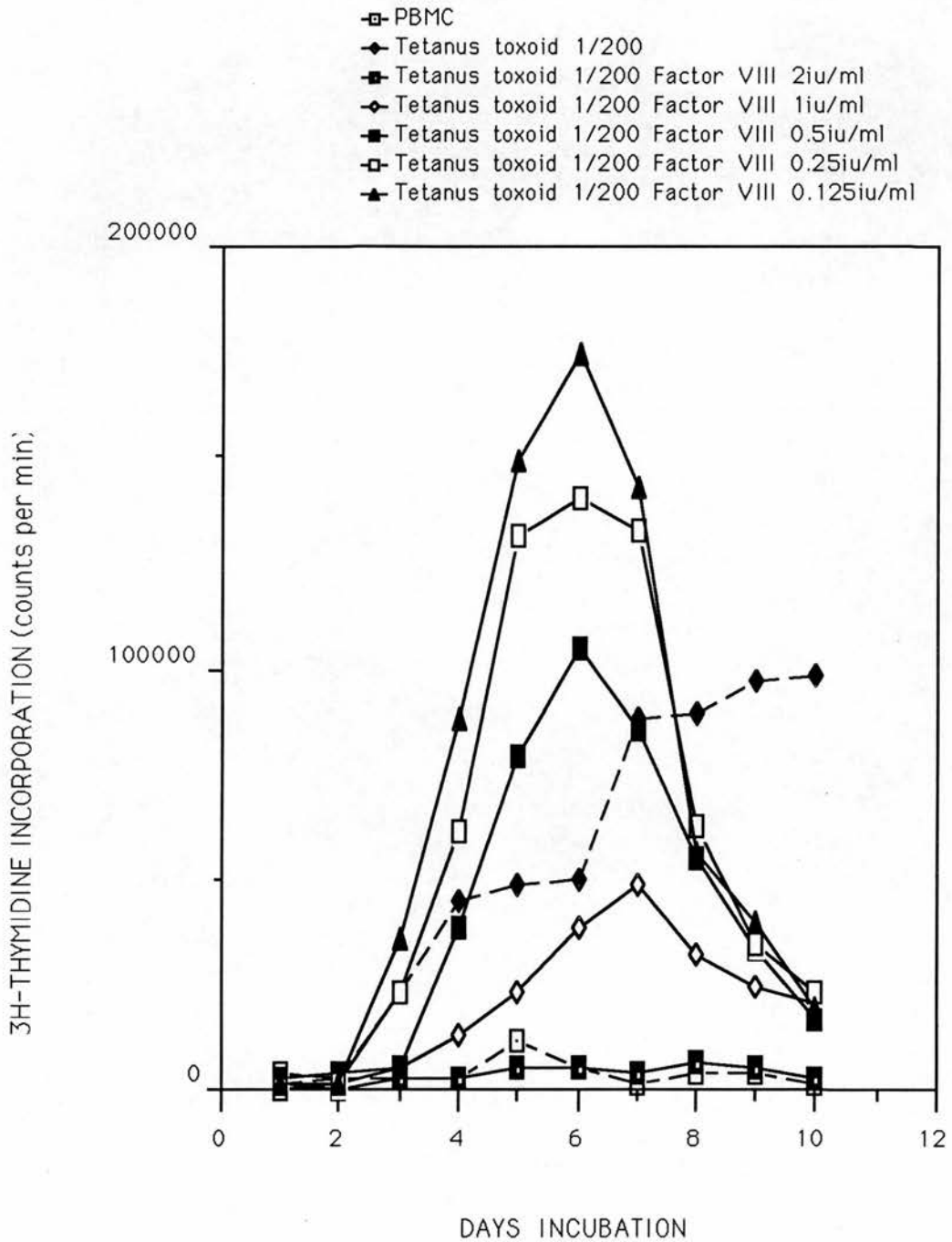


Fig 23
Lymphocyte proliferation in the presence of SNBTS Z8-6026 following stimulation with the recall antigen tetanus toxoid. Total protein included per assay ranged from 0.28 mg/ml to 4.41 mg/ml.

3 Effect of SNBTS factor VIII batch Z8-6026 on lymphocyte proliferation following stimulation with pokeweed mitogen

Lymphocyte proliferation in response to PWM was completely inhibited by SNBTS factor VIII batch Z8-6026 when present at 4iu/ml. Over a lower range of concentrations this batch of concentrate enhanced ^3H -thymidine incorporation in response to PWM stimulation (Fig 24).

In the absence of mitogen or antigen stimulation SNBTS factor VIII batch Z8-6026 did not stimulate peripheral blood mononuclear cells.

4 Effect of factor VIII concentrate on lymphocyte proliferation following stimulation with irradiated allogeneic lymphoblastoid cells

4.1 ^3H -thymidine incorporation

Seven of seven batches of SNBTS Z8 factor VIII concentrate tested enhanced lymphocyte proliferation in response to irradiated allogeneic lymphoblastoid cells in a weak one-way mixed lymphocyte reaction (Fig 25).

Peripheral blood mononuclear cells did not respond to SNBTS intermediate purity factor VIII concentrate in the absence of allogeneic stimulator cells.

In a comparative series SNBTS Z8 and SNBTS S8 (prototype higher purity factor VIII) enhanced lymphocyte proliferation in a weak one-way mixed lymphocyte reaction (Fig 26, 27).

In contrast, SNBTS control buffer, high purity (Bio-transfusion) and monoclonal antibody purified (Monoclone-P) factor VIII preparations did not alter the response to allogeneic stimulator cells (Fig 28, 29, 30).

Factor VIII concentrates were included at equivalent dilutions of factor VIII activity.

High purity concentrates Profilate and Octa-Pharma V.I.250 caused little or no enhanced proliferation in a weak one-way mixed lymphocyte reaction (Fig 31, 32)

SNBTS Z8-6026

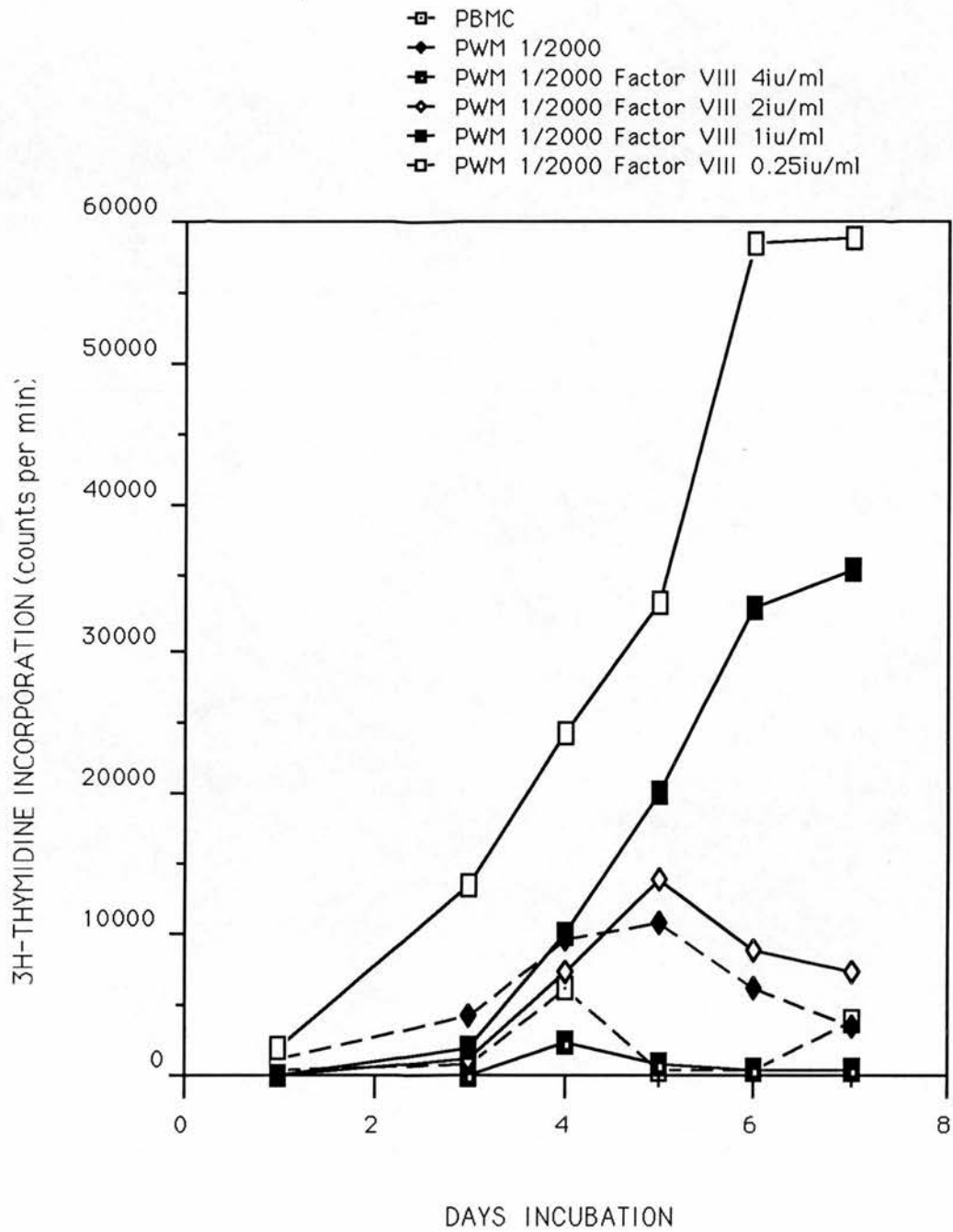


Fig 24
Effect of SNBTS factor VIII batch Z8-6026 on PBMC proliferation following stimulation with PWM. Total protein included per assay ranged from 0.55 mg/ml to 8.82 mg/ml.

SNBTS Z8-81562

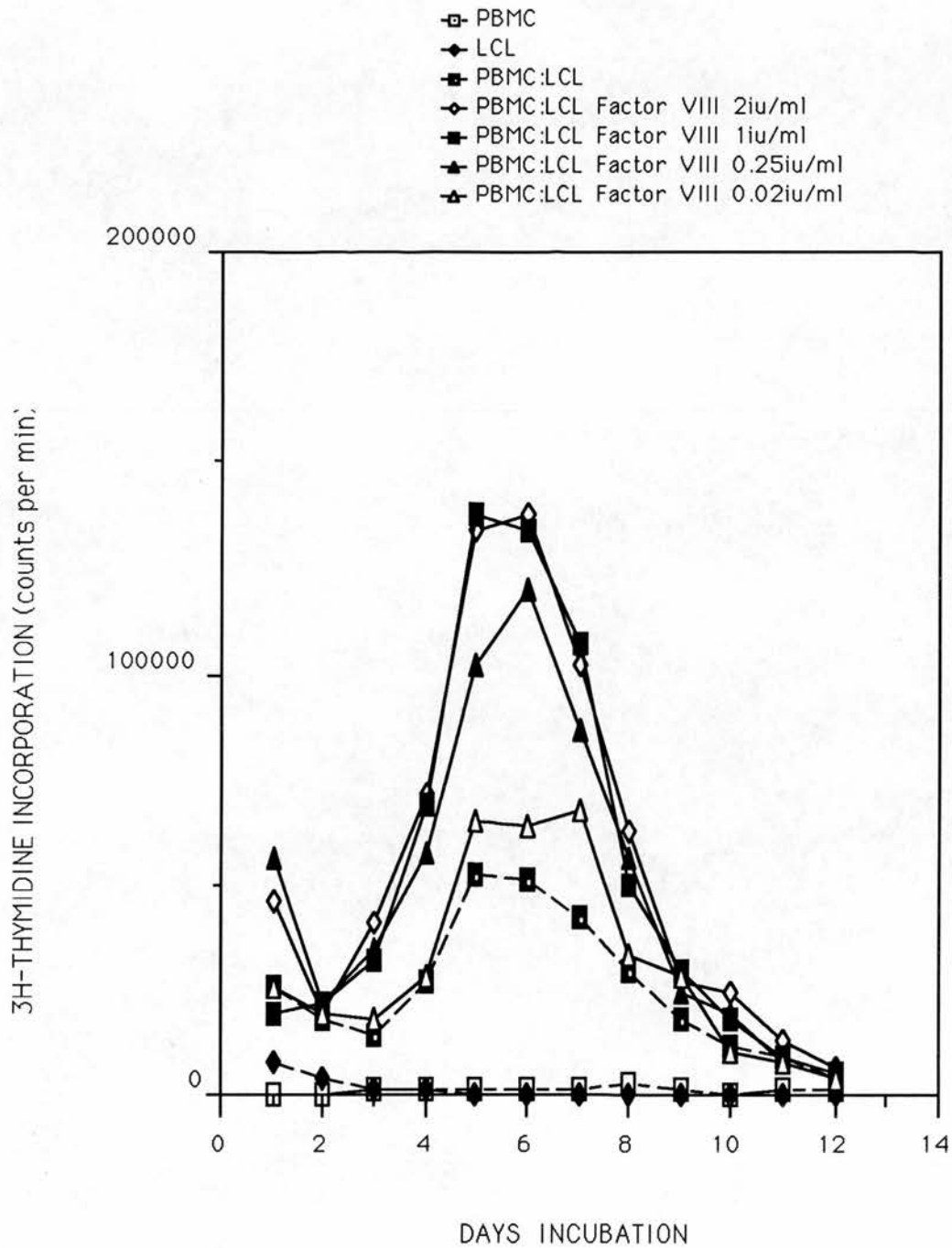


Fig 25
Enhanced lymphocyte proliferation in the presence of SNBTS Z8 factor VIII concentrate. PBMC were stimulated with irradiated allogeneic lymphoblastoid cells in the presence of various concentrations of factor VIII. Total protein included per assay ranged from 0.04 mg/ml to 4.18 mg/ml.

SNBTS Z8-92520
INTERMEDIATE PURITY FACTOR VIII

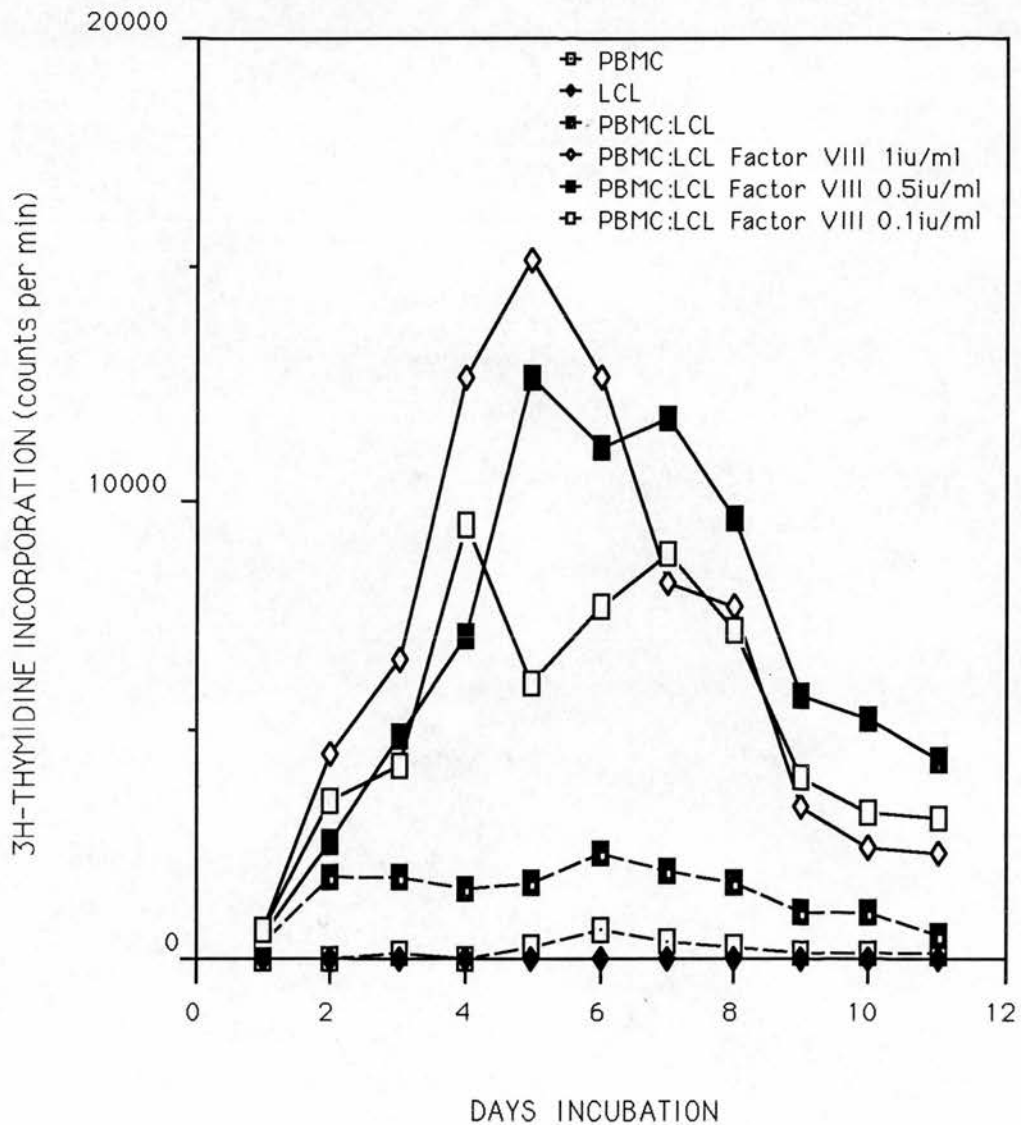


Fig 26
Effect of SNBTS Z8 factor VIII on lymphocyte proliferation in a weak one way mixed lymphocyte reaction. Enhanced proliferation was evident when factor VIII was included at 0.1 iu/ml to 1.0 iu/ml (total protein 0.22 mg/ml to 2.18 mg/ml).

SNBTS S8-0006
PROTOTYPE HIGH PURITY FACTOR VIII

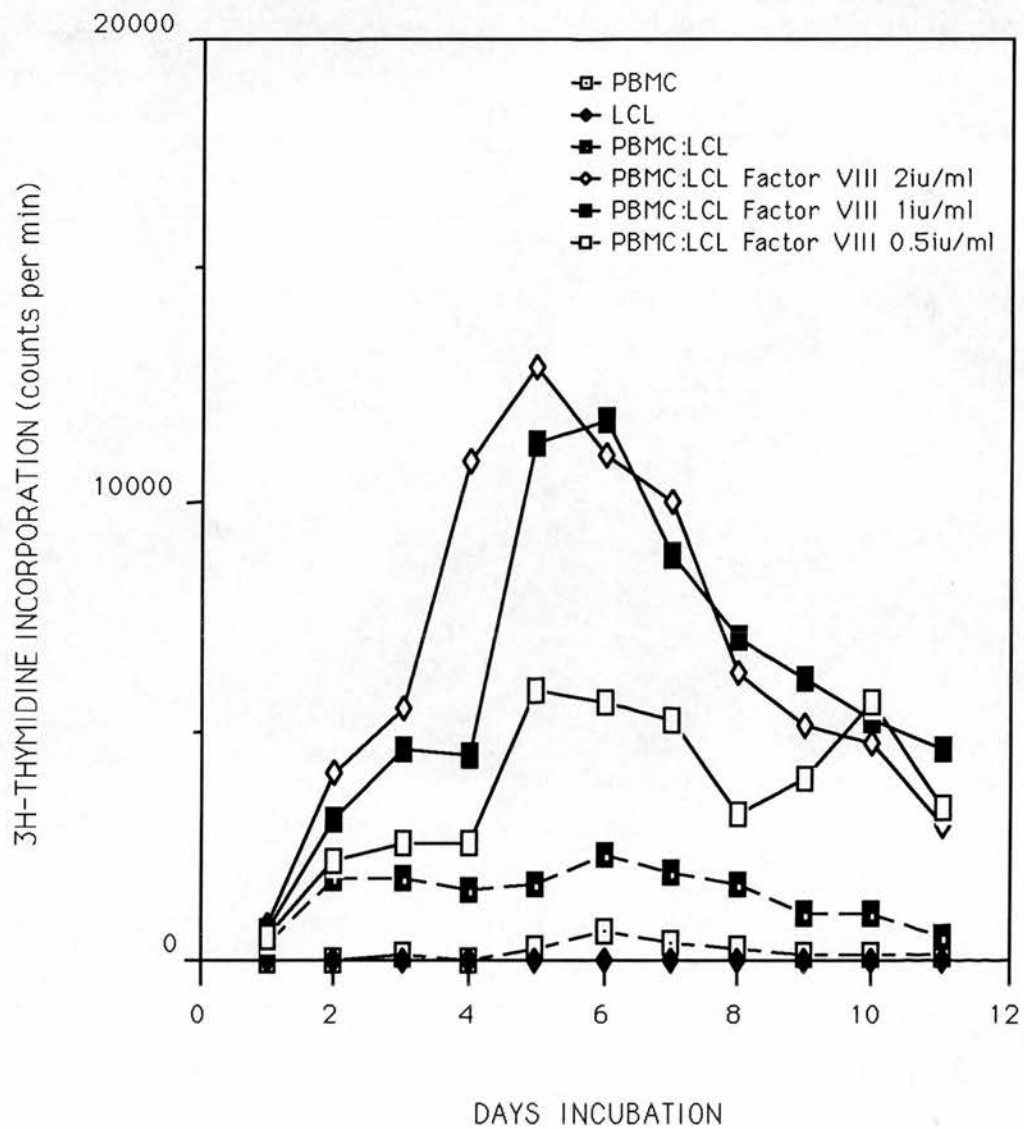


Fig 27
 Effect of prototype high purity SNBTS S8 factor VIII concentrate on lymphocyte proliferation in response to irradiated allogeneic stimulator cells. Total protein included ranged from 0.38 mg/ml to 1.53 mg/ml.

SNBTS CONTROL BUFFER

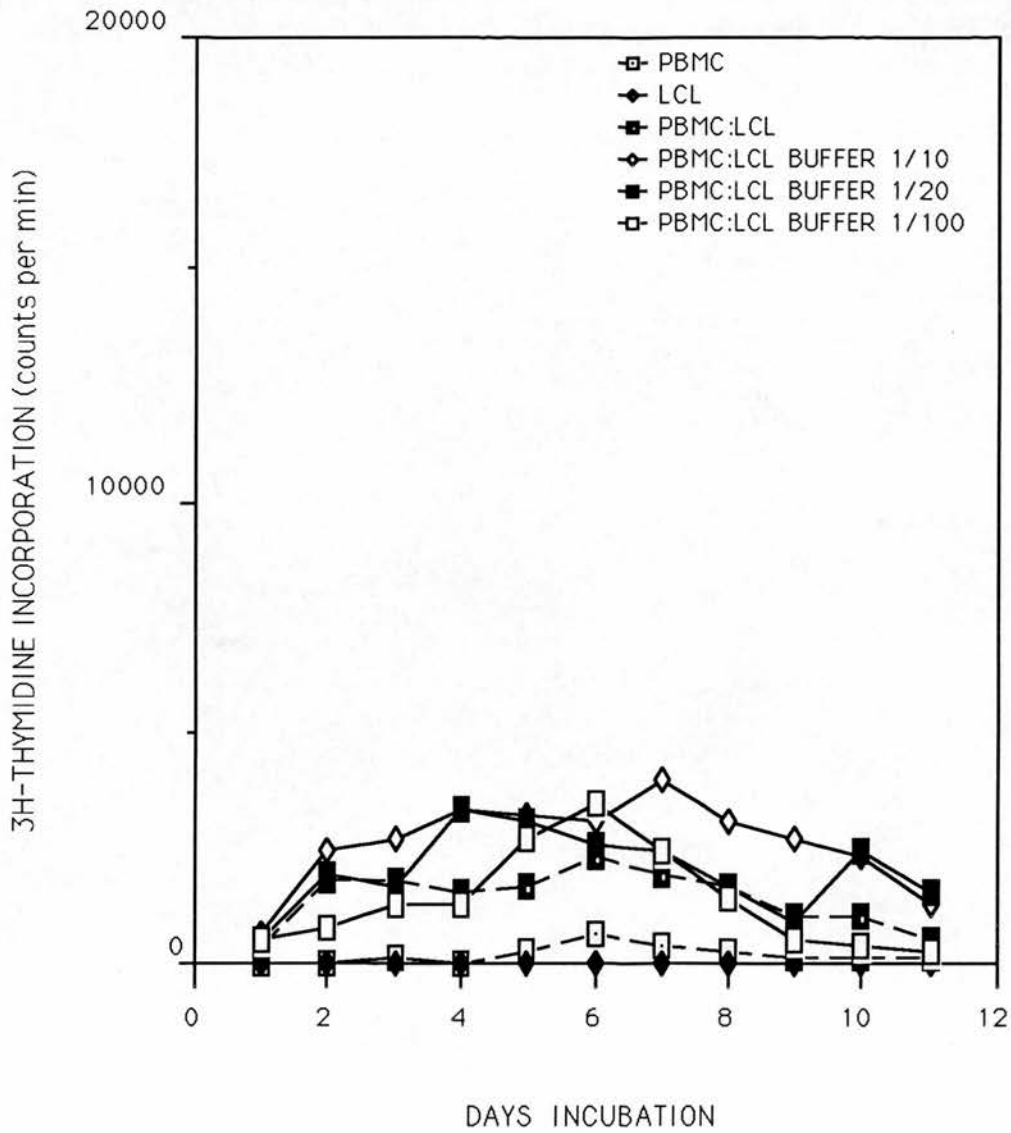


Fig 28

The proliferative response to allogeneic stimulator cells was not affected by the presence of SNBTS control buffer. Dilutions of buffer included were equivalent to the dilutions of SNBTS factor VIII concentrate assayed.

BIO-TRANSFUSION

HIGH PURITY FACTOR VIII

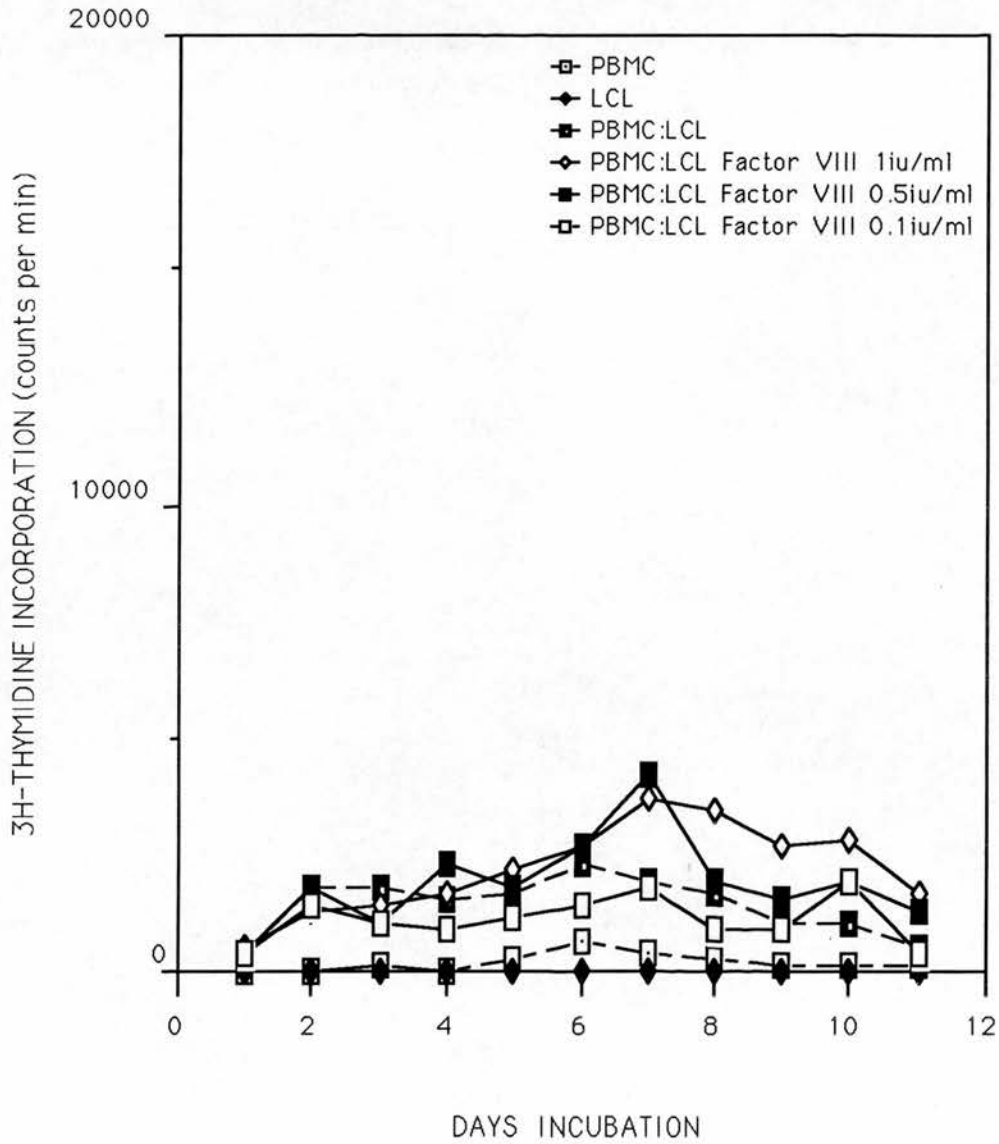


Fig 29

High purity Bio-transfusion factor VIII concentrate had no effect on PBMC proliferation in response to irradiated allogeneic stimulator cells. Total protein included per assay ranged from 5.6×10^{-4} mg/ml to 5.6×10^{-3} mg/ml.

MONOCLATE-P
MONOCLONAL ANTIBODY PURIFIED FACTOR VIII

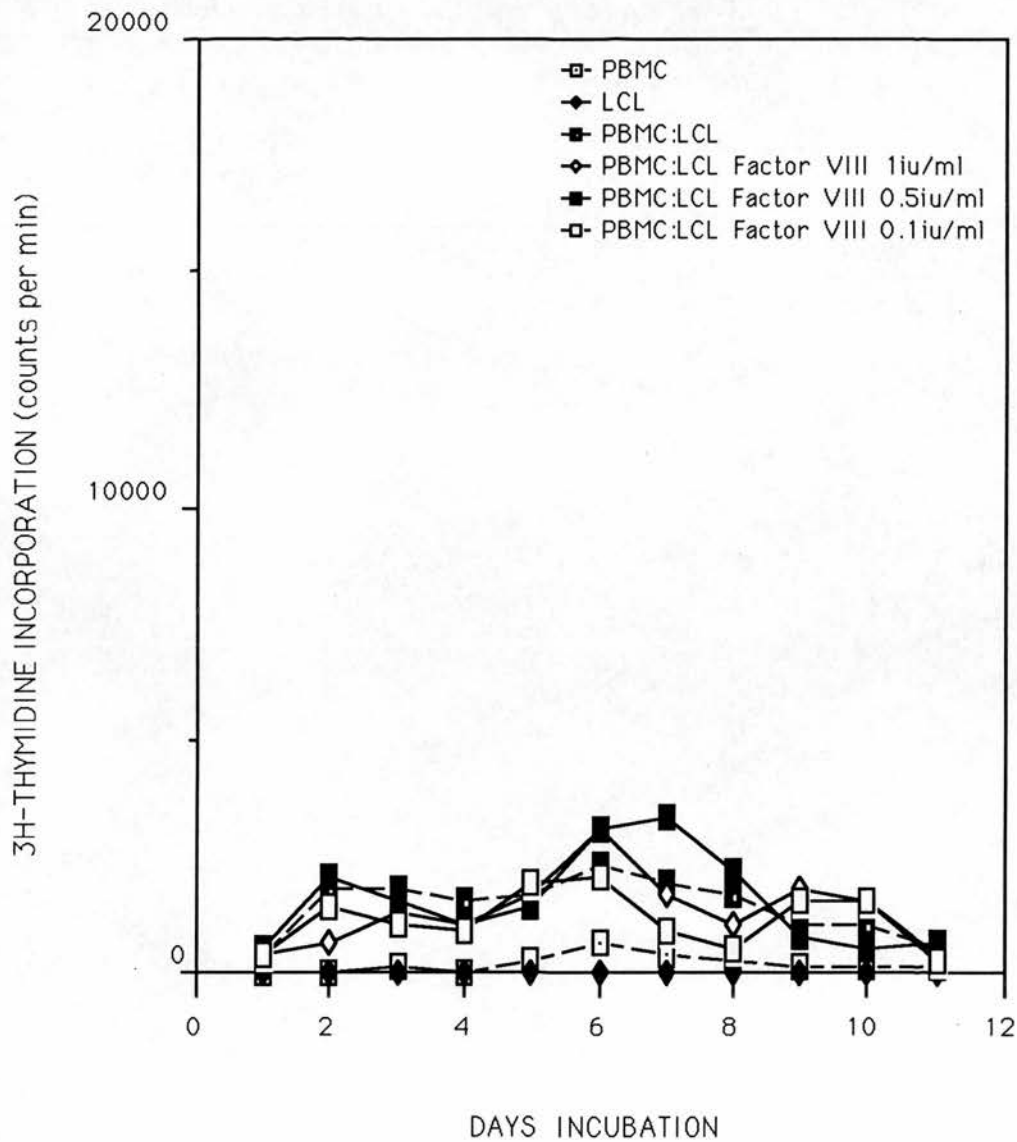


Fig 30

The proliferative response to allogeneic stimulator cells was not affected by the presence of monoclonal antibody purified Monoclote-P factor VIII concentrate. Total protein included per assay ranged from 7.5×10^{-3} mg/ml to 7.5×10^{-2} mg/ml.

PROFILATE

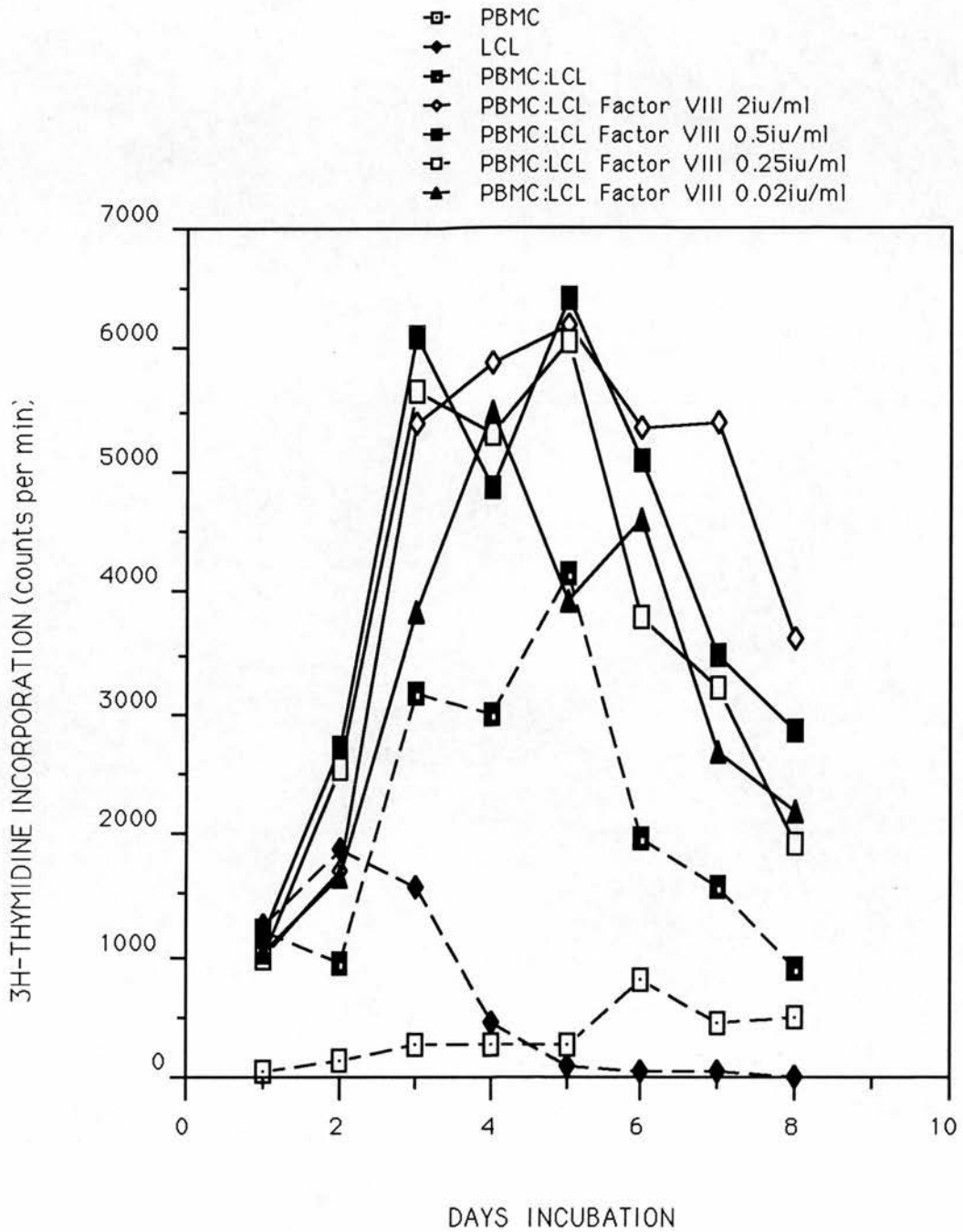


Fig 31
Effect of high purity Profilate factor VIII concentrate on lymphocyte proliferation in the mixed lymphocyte reaction. Total protein included per assay ranged from 4.5×10^{-3} mg/ml to 0.45 mg/ml.

OCTA-PHARMA V.I.250

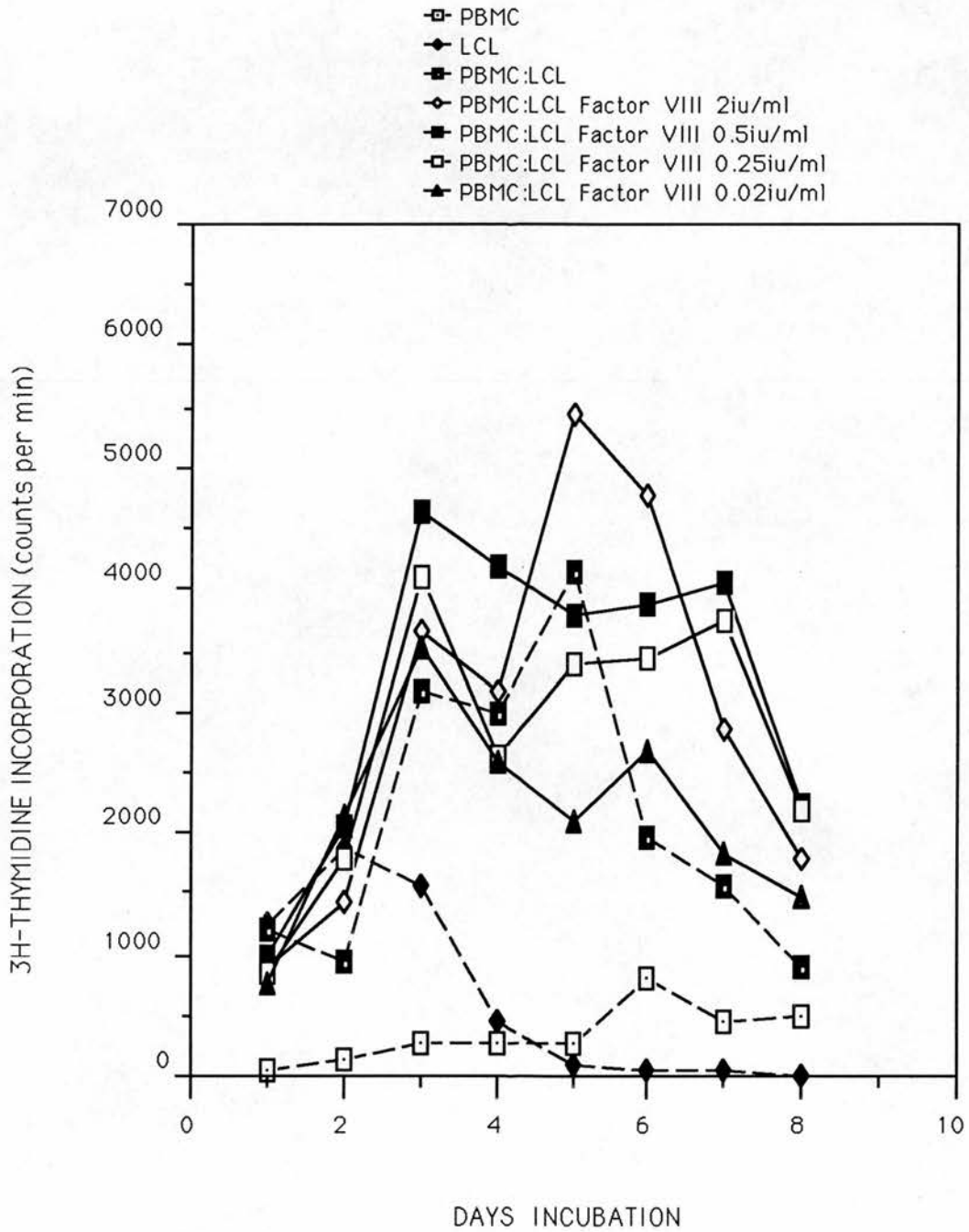


Fig 32

Effect of high purity Octa-Pharma V.I. 250 factor VIII concentrate on lymphocyte proliferation in response to irradiated allogeneic stimulator cells. Total protein included per assay ranged from 4×10^{-3} mg/ml to 0.4 mg/ml.

The monoclonal antibody purified factor VIII concentrate, Hemofil-M did not significantly enhance ^3H -thymidine incorporation in response to allogeneic stimulator cells (Fig 33).

Three of three batches of Bio Products Laboratory 8Y factor VIII concentrate enhanced lymphocyte proliferation in a weak one-way mixed lymphocyte reaction, but to a lesser extent than SNBTS intermediate purity factor VIII (Fig 34).

When incubated at high concentrations factor IX concentrate manufactured by the Scottish National Blood Transfusion Service enhanced lymphocyte proliferation in this assay system (Fig 35).

5 Characterisation of cells in mixed lymphocyte reactions

Cells proliferating in response to irradiated allogeneic stimulator cells and SNBTS Z8 factor VIII were characterised by flow cytometry.

Proliferating cells included CD 4⁺ and CD 8⁺ T lymphocytes. Seven percent of cells analysed expressed the B cell marker CD 19. Both T cell subsets expressed a range of cell surface activation markers including CD 71, CD 25 and HLA.DR. A smaller proportion of T cells expressed CDw 49, an antigen mainly found on platelets and activated and long-term cultivated T cells. The intercellular adhesion molecule, ICAM-1 (CD 54), was expressed on 70% and 49% of CD 4⁺ and CD 8⁺ cells respectively. A large proportion of T cells expressed the memory T cell marker CD 45RO (Table 4).

6 Physical manipulation of SNBTS intermediate purity factor VIII concentrate

6.1 Freezing, filtering and defibrinating factor VIII

Peripheral blood mononuclear cell transformation in response to allogeneic lymphoblastoid stimulator cells was enhanced in the presence of fresh, freeze-thawed, membrane filtered and defibrinated SNBTS Z8 factor VIII concentrate (Fig 36, 37, 38).

HEMOFIL-M

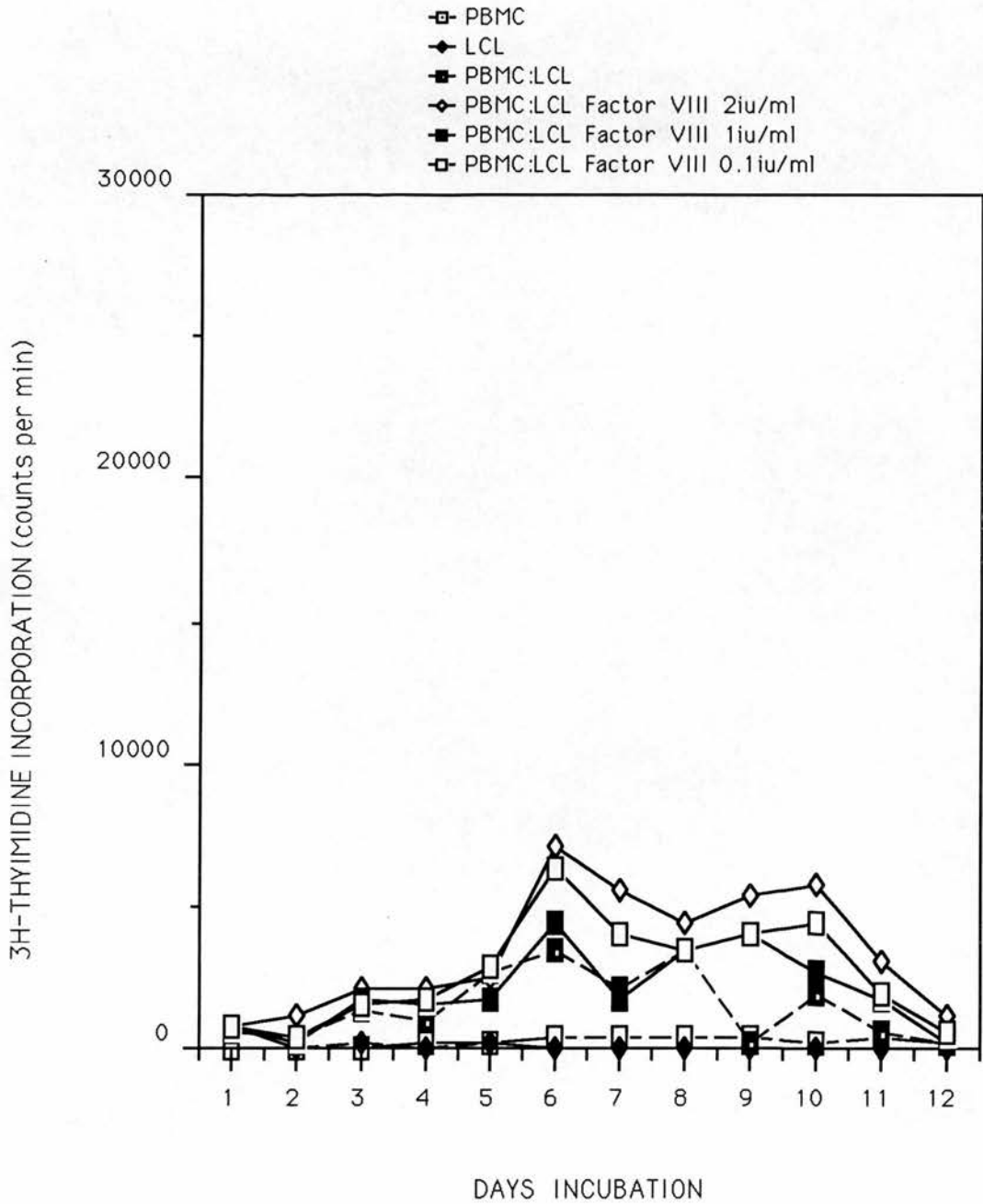


Fig 33
Effect of monoclonal antibody purified Hemofil-M factor VIII concentrate on lymphocyte proliferation in the mixed lymphocyte reaction.

**BIO PRODUCTS LABORATORY
FHC 0110**

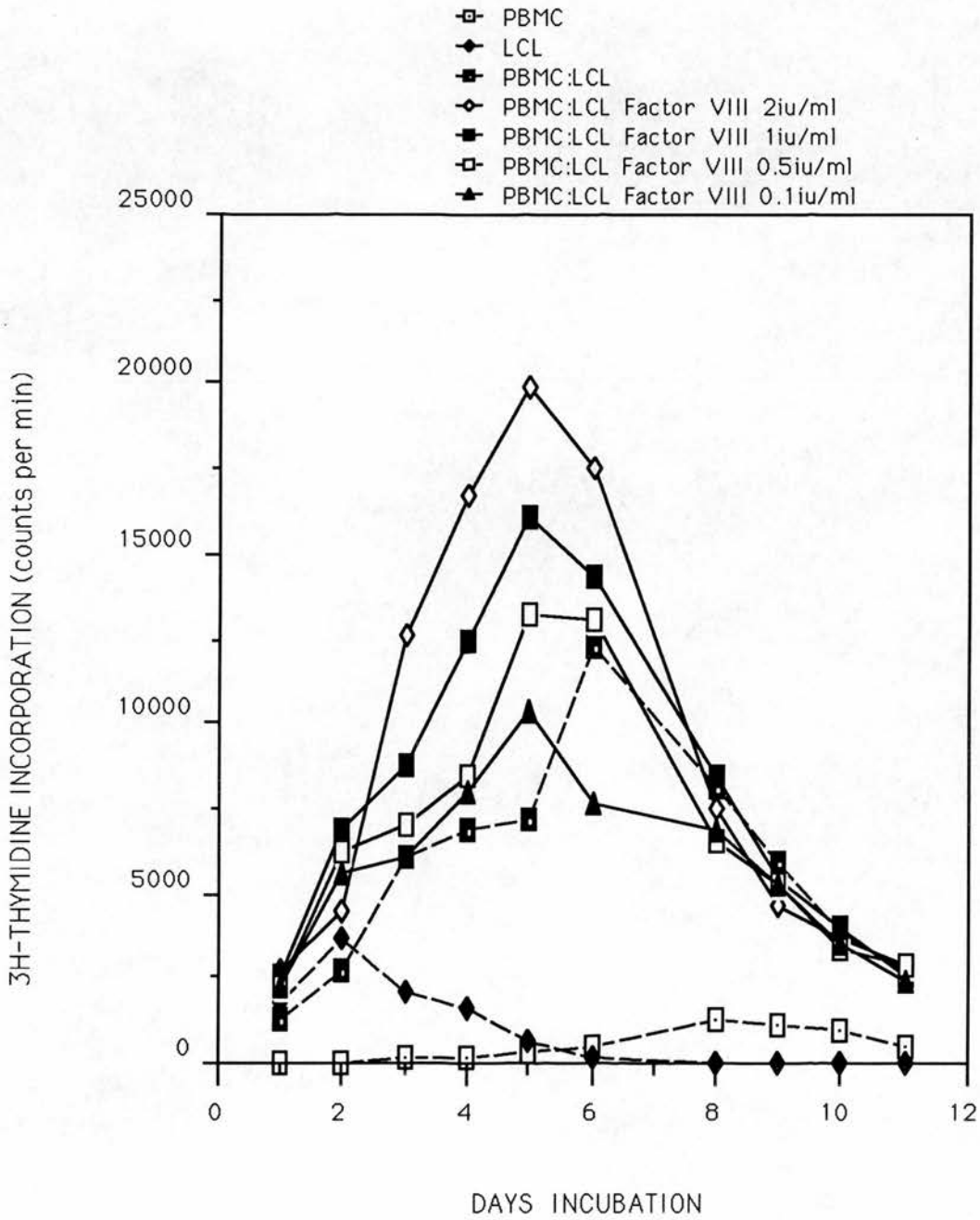


Fig 34
Intermediate purity Bio Products Laboratory factor VIII concentrate enhanced lymphocyte proliferation in response to irradiated allogeneic stimulator cells when present at high concentrations. Total protein included per assay ranged from 0.03 mg/ml to 0.51 mg/ml.

SNBTS **FACTOR IX 90560**

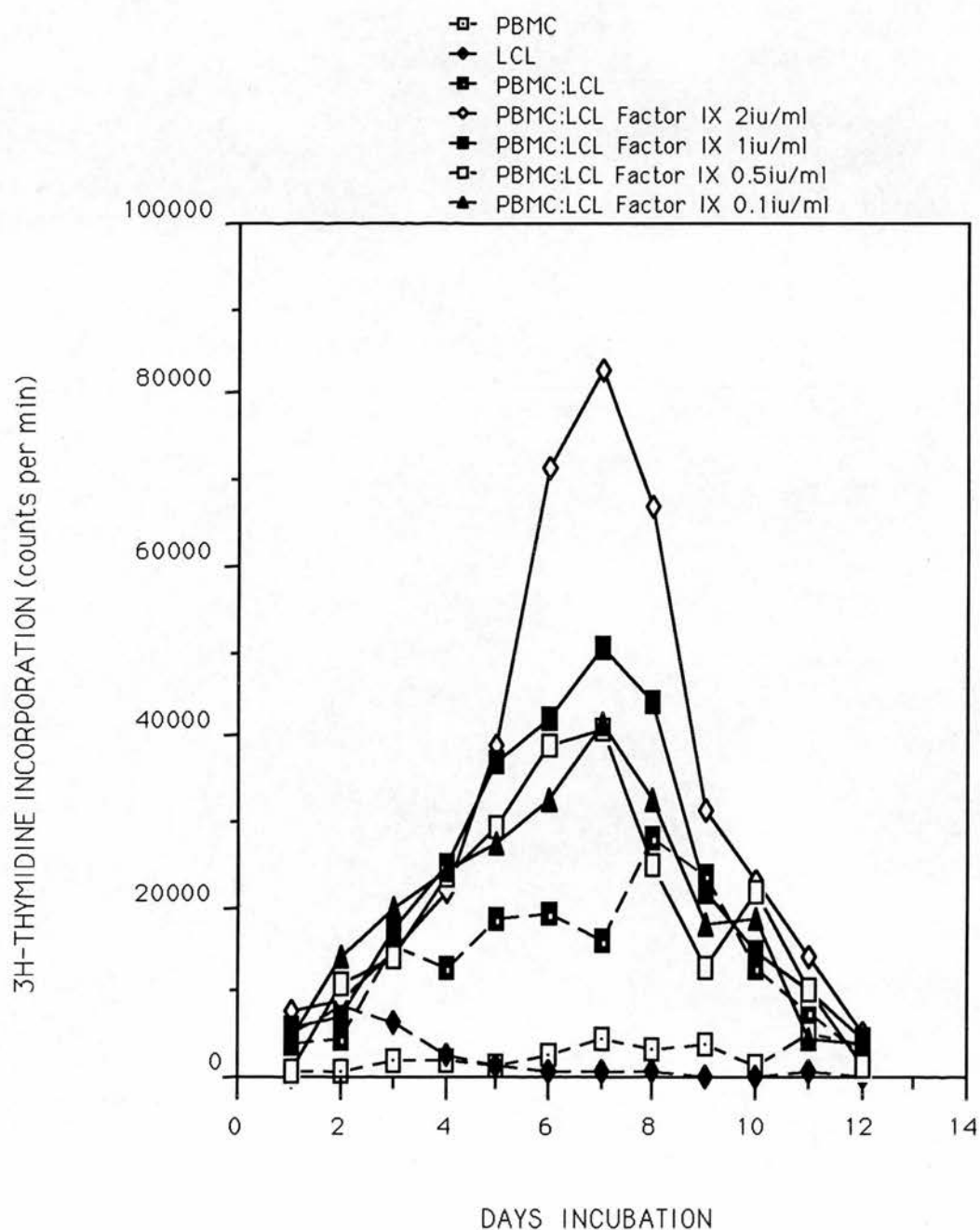


Fig 35
Effect of SNBTS factor IX concentrate on lymphocyte proliferation in the mixed lymphocyte reaction. Total protein included per assay ranged from 0.04 mg/ml to 0.88 mg/ml.

% positive cells

mAB	CD4	CD8
CD71	52	49
CD25	63	42
HLA.DR	45	48
CDw49	12	23
CD54	70	49
CD45RO	63	55
CD3	86	89
CD19	2	1
CD29	74	53

Responding cells were harvested and characterised by two colour flow cytometry, as described in text, 5 days after establishment of cultures. PBMC were stimulated with irradiated allogeneic lymphoblastoid cells in the presence of 2iu/ml SNBTS factor VIII.

Table 4
Characterisation of cells proliferating in response to irradiated allogeneic stimulator cells and SNBTS Z8 factor VIII concentrate by flow cytometry.

SNBTS Z8-92520 **FREEZE-THAWED**

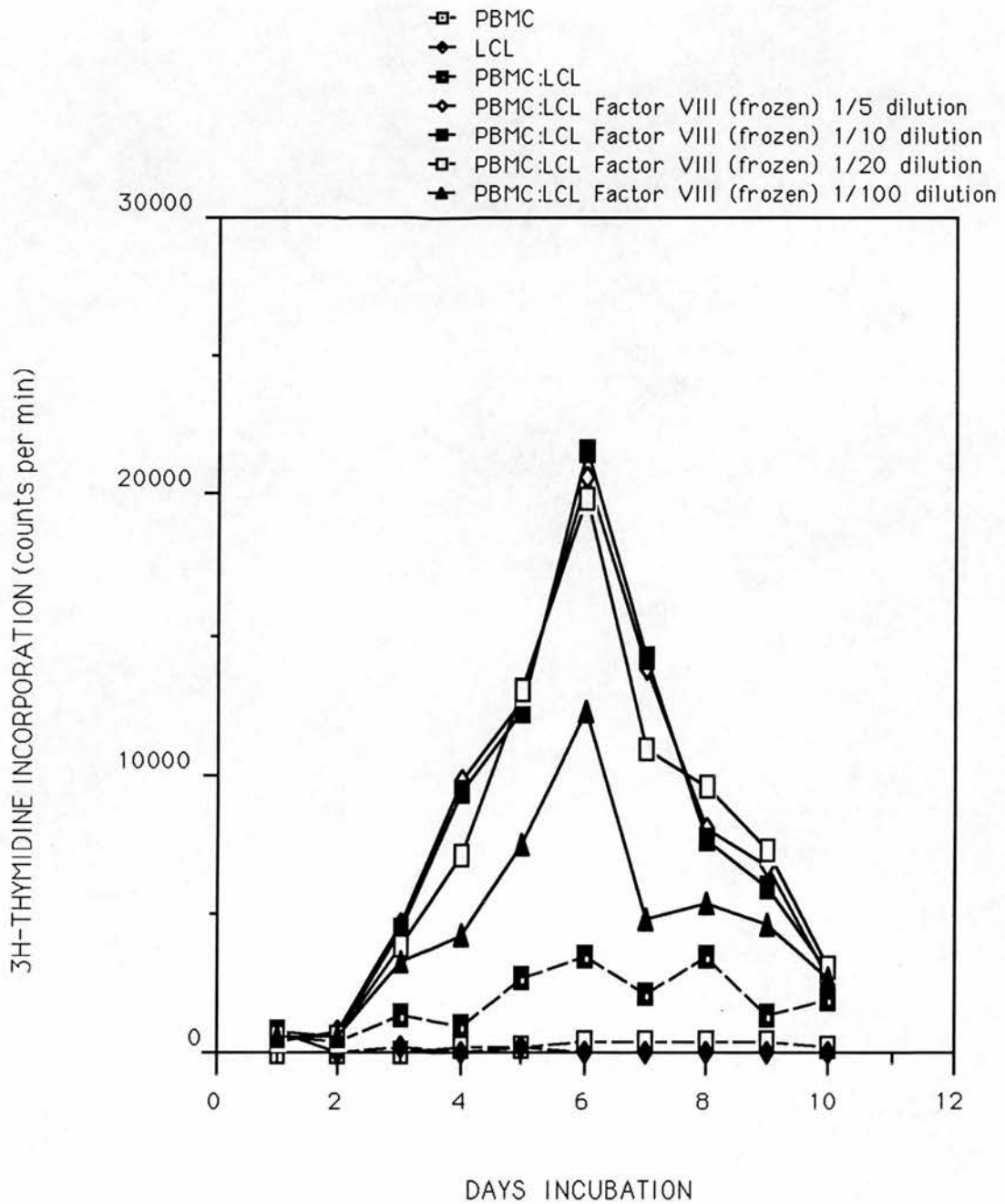


Fig 36

Freeze-thawing has no effect on the co-mitogenic properties of SNBTS Z8 factor VIII concentrate in the one way mixed lymphocyte reaction.

**SNBTS Z8-92340
FILTERED**

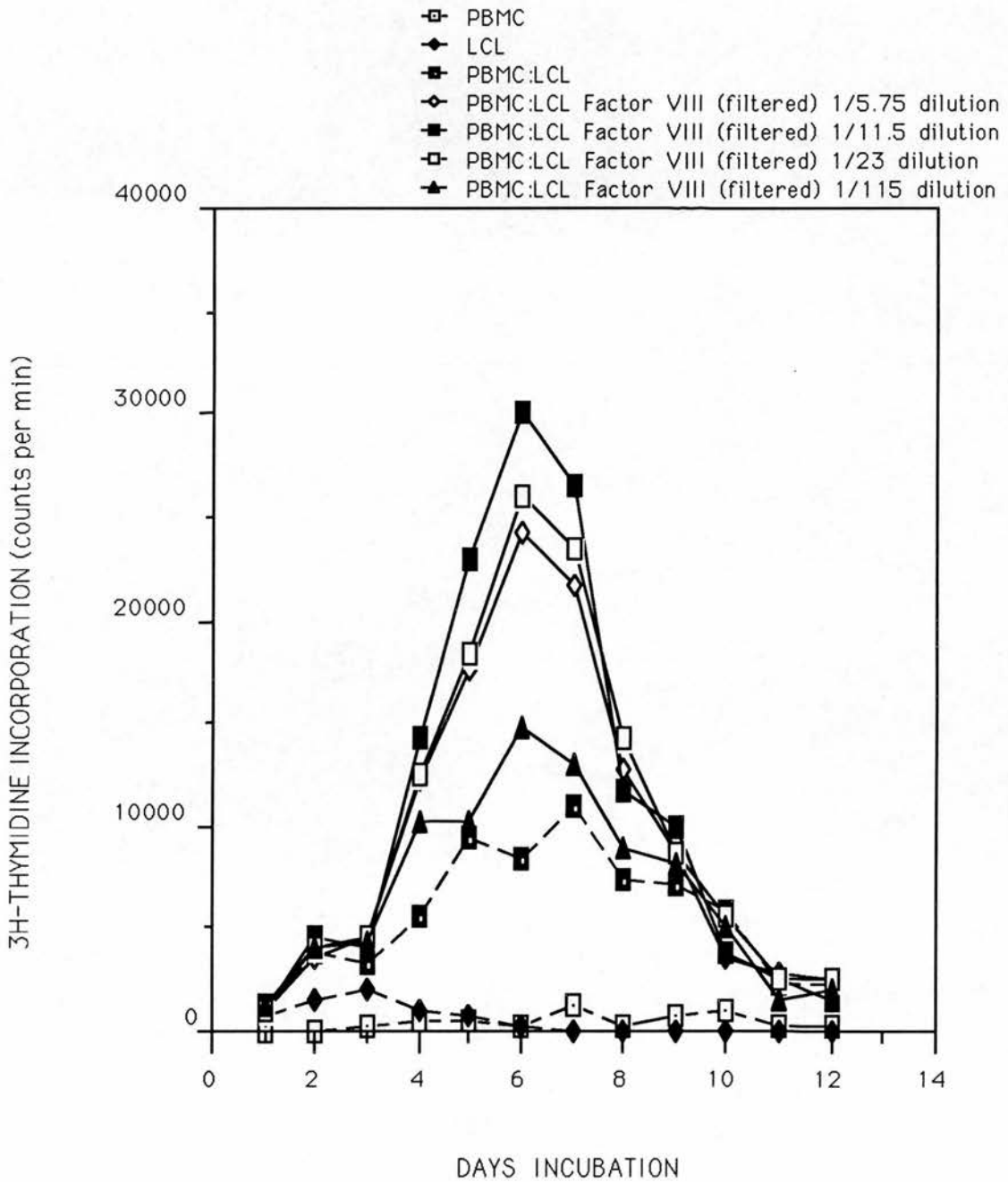


Fig 37

The effect of syringe filtering (0.2 μ m pore size) SNBTS Z8 factor VIII prior to inclusion in the one way mixed lymphocyte reaction. Enhanced lymphocyte proliferation in response to irradiated allogeneic cells occurs in the presence of filtered factor VIII concentrate.

**SNBTS Z8-92340
DEFIBRINATED**

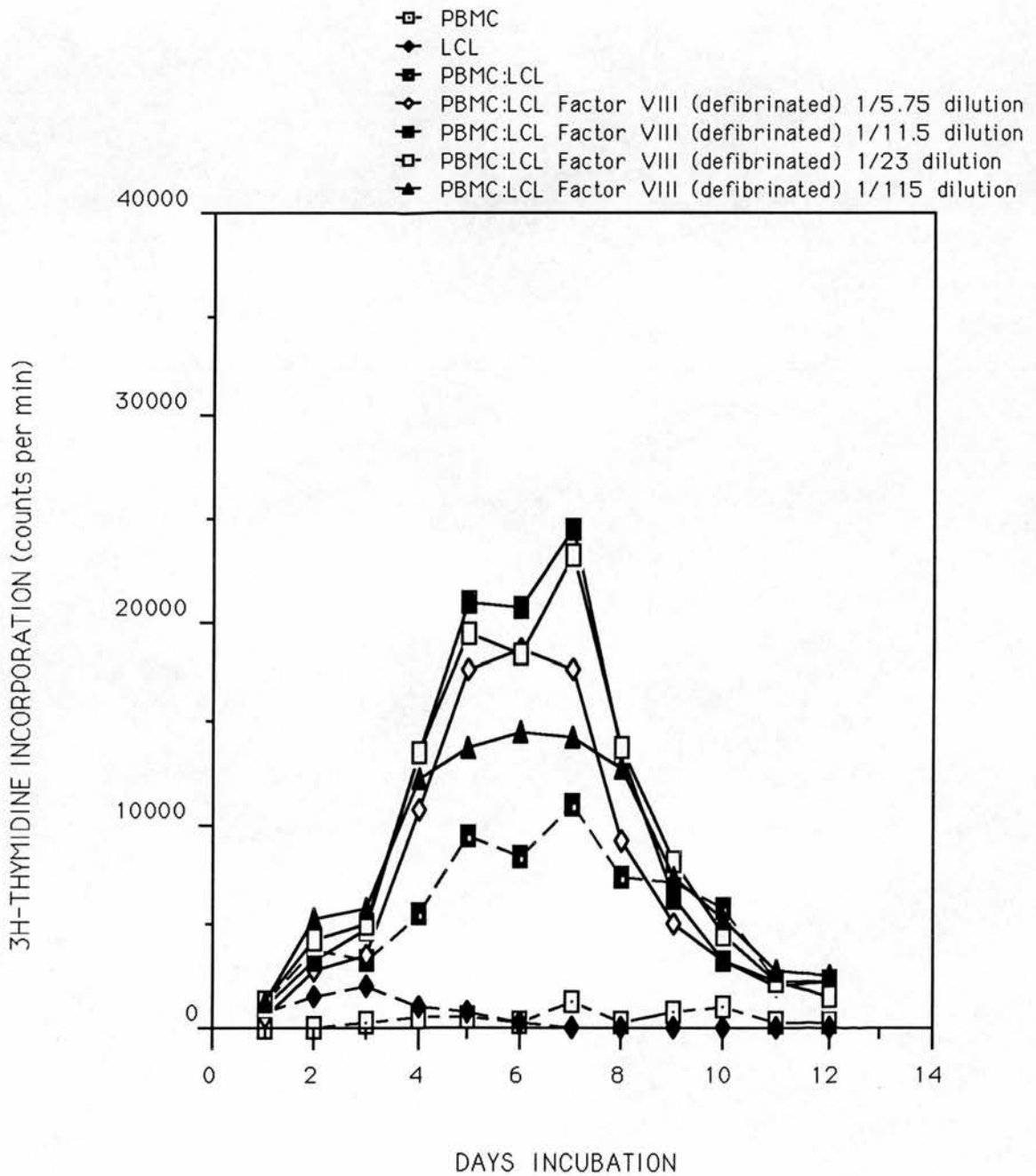


Fig 38
Enhanced lymphocyte proliferation in response to irradiated allogeneic stimulator cells in the presence of defibrinated SNBTS Z8 factor VIII.

6.2 Molecular weight cut off filters

In two series defibrinated factor VIII was filtered through Amicon Centriprep ultrafiltration devices to give broad molecular weight fractions.

In the first series, when fractions were assayed in a weak one-way mixed lymphocyte reaction, the enhancing activity was confined to fractions of >100 KDa molecular weight (Fig 39). The results are expressed in terms of cumulative total ^3H -thymidine incorporation for the period of the assay, in counts per minute.

On further filter fractionation the activity was found to be in the fraction containing components of molecular weight >30 KDa (Fig 40).

7 Fibronectin

Fibronectin is present at a considerable concentration in SNBTS Z8 and S8 concentrates. Human fibronectin did not enhance lymphocyte proliferation in response to allogeneic lymphoblastoid cells when present at concentrations representative of those in factor VIII preparations (Fig 41).

Peripheral blood mononuclear cells did not respond to fibronectin in the absence of irradiated allogeneic stimulator cells. The fibronectin preparation was not cytotoxic to peripheral blood mononuclear cells.

8 Preincubation of SNBTS Z8 factor VIII with peripheral blood mononuclear cells or irradiated lymphoblastoid cells

Significant enhancement of lymphocyte proliferation following stimulation with irradiated allogeneic lymphoblastoid cells only occurred if factor VIII concentrate was present throughout the culture period (Fig 42).

Peripheral blood mononuclear cells incubated with factor VIII overnight, then stimulated with irradiated allogeneic lymphoblastoid cells responded weakly, with limited ^3H -thymidine incorporation. Transformation was limited when irradiated lymphoblastoid cells were incubated with factor VIII overnight and then used to stimulate peripheral blood mononuclear cells. Similarly, peripheral blood

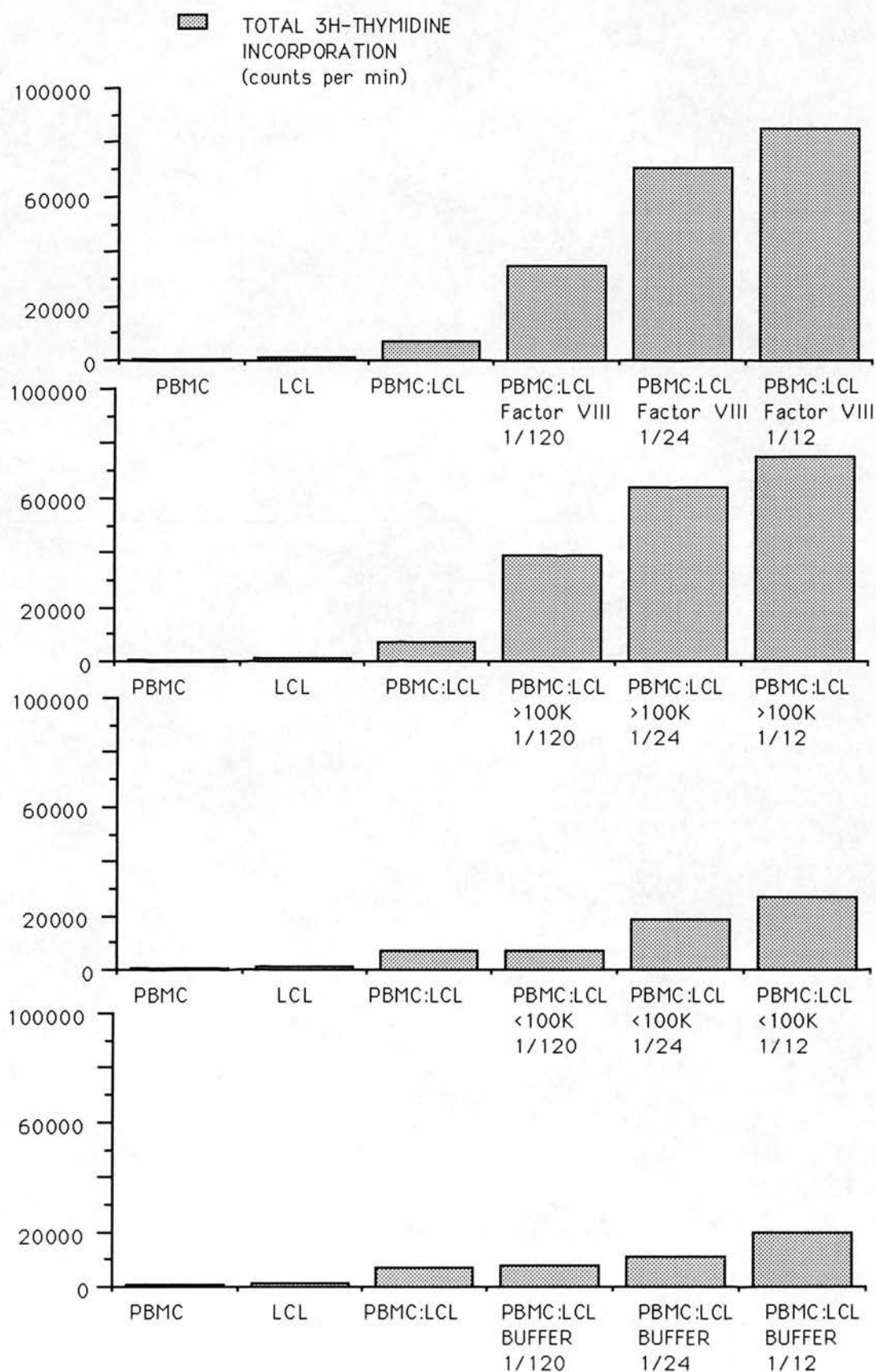


Fig 39

Molecular weight cut off filters were used to crudely fractionate defibrinated SNBTS Z8 factor VIII. Fractions were assayed in the one way mixed lymphocyte reaction and results expressed in terms of cumulative total 3H-thymidine incorporation (counts per min) for the period of the assay.

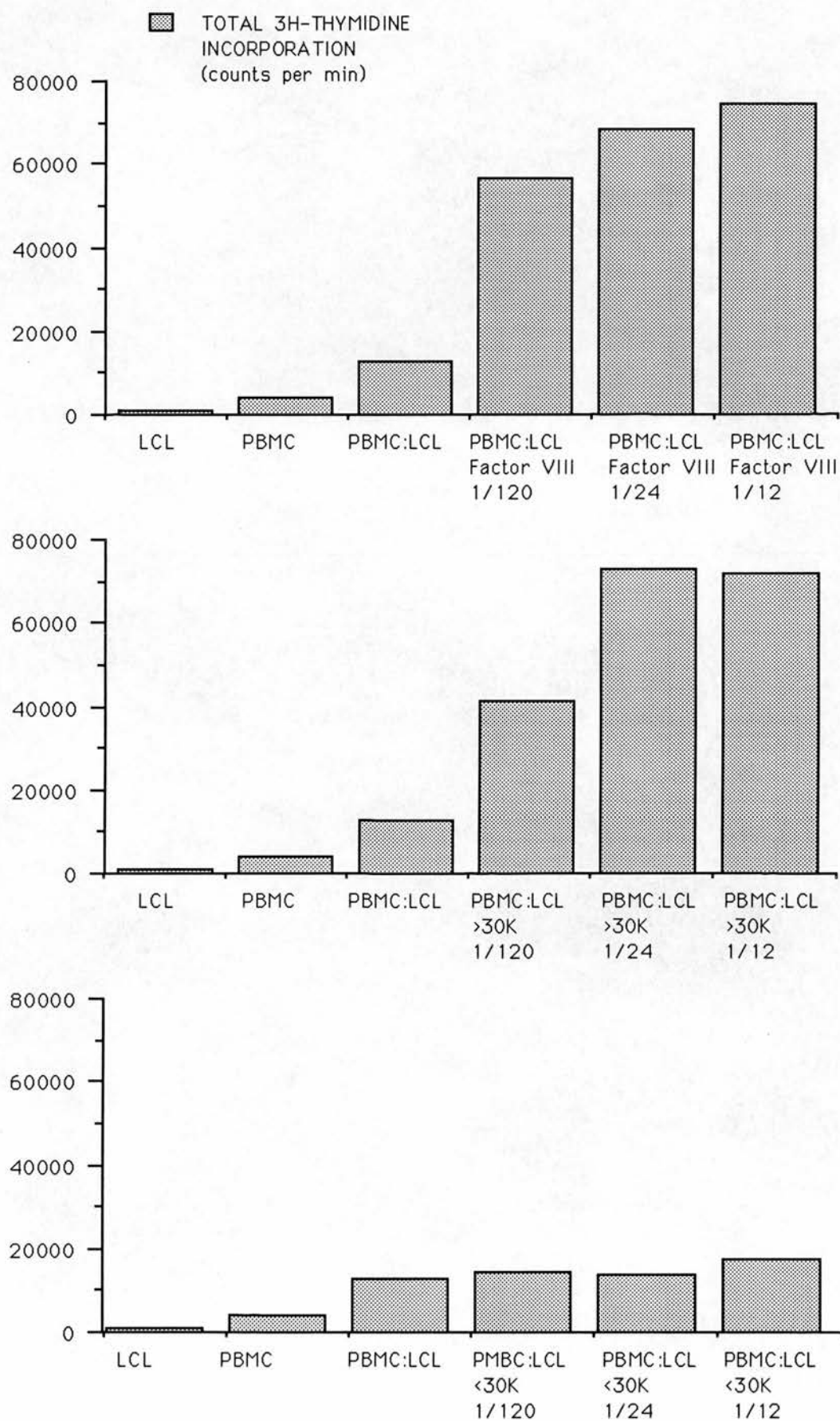


Fig 40 (i)

Molecular weight cut off filters were used to crudely fractionate defibrinated SNBTS Z8 factor VIII. Fractions were assayed in the one way mixed lymphocyte reaction and results expressed in terms of cumulative total 3H-thymidine incorporation (counts per min) for the period of the assay.

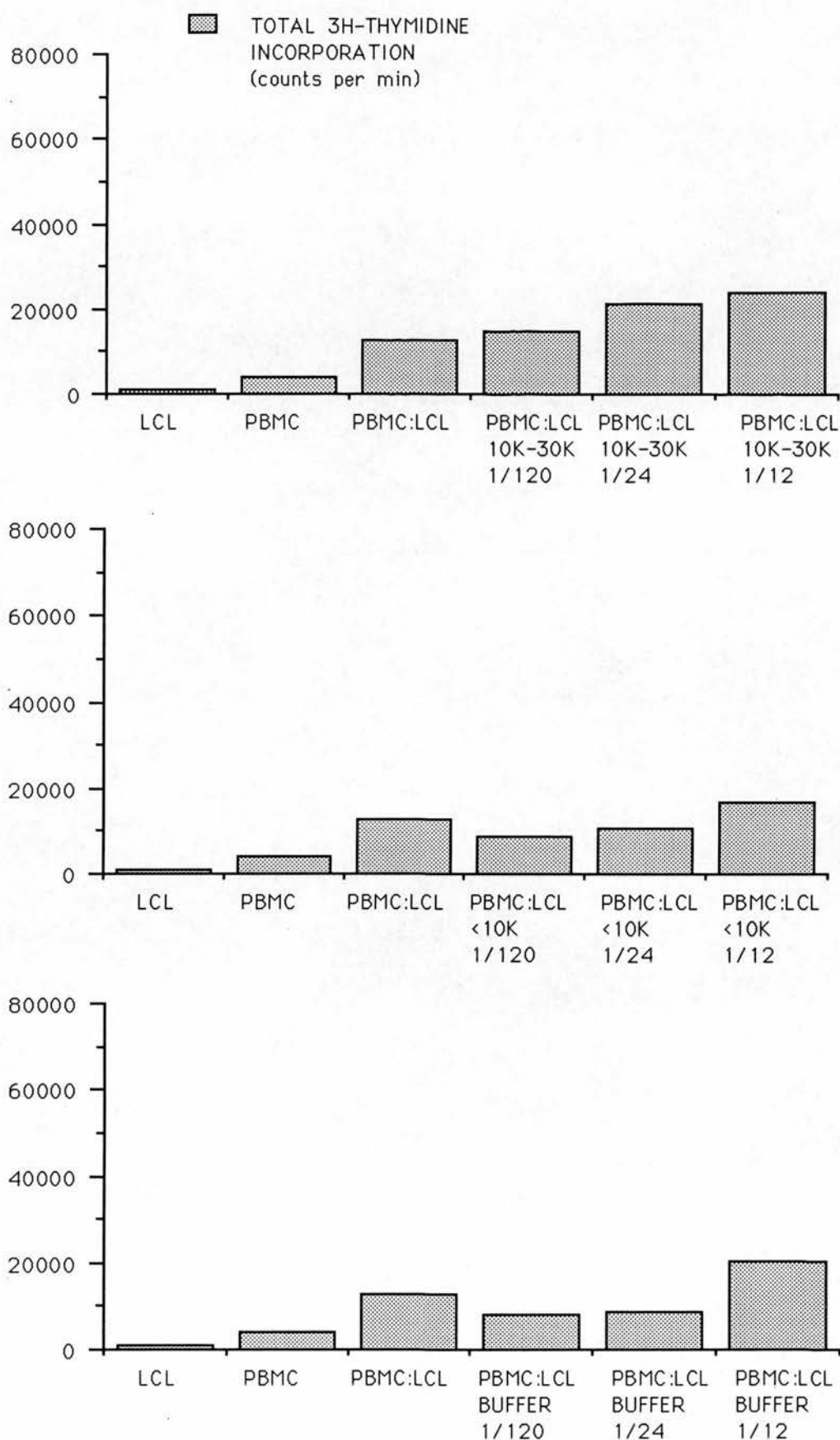


Fig 40 (ii)

Molecular weight cut off filters were used to crudely fractionate defibrinated SNBTS Z8 factor VIII. Fractions were assayed in the one way mixed lymphocyte reaction and results expressed in terms of cumulative total 3H-thymidine incorporation (counts per min) for the period of the assay.

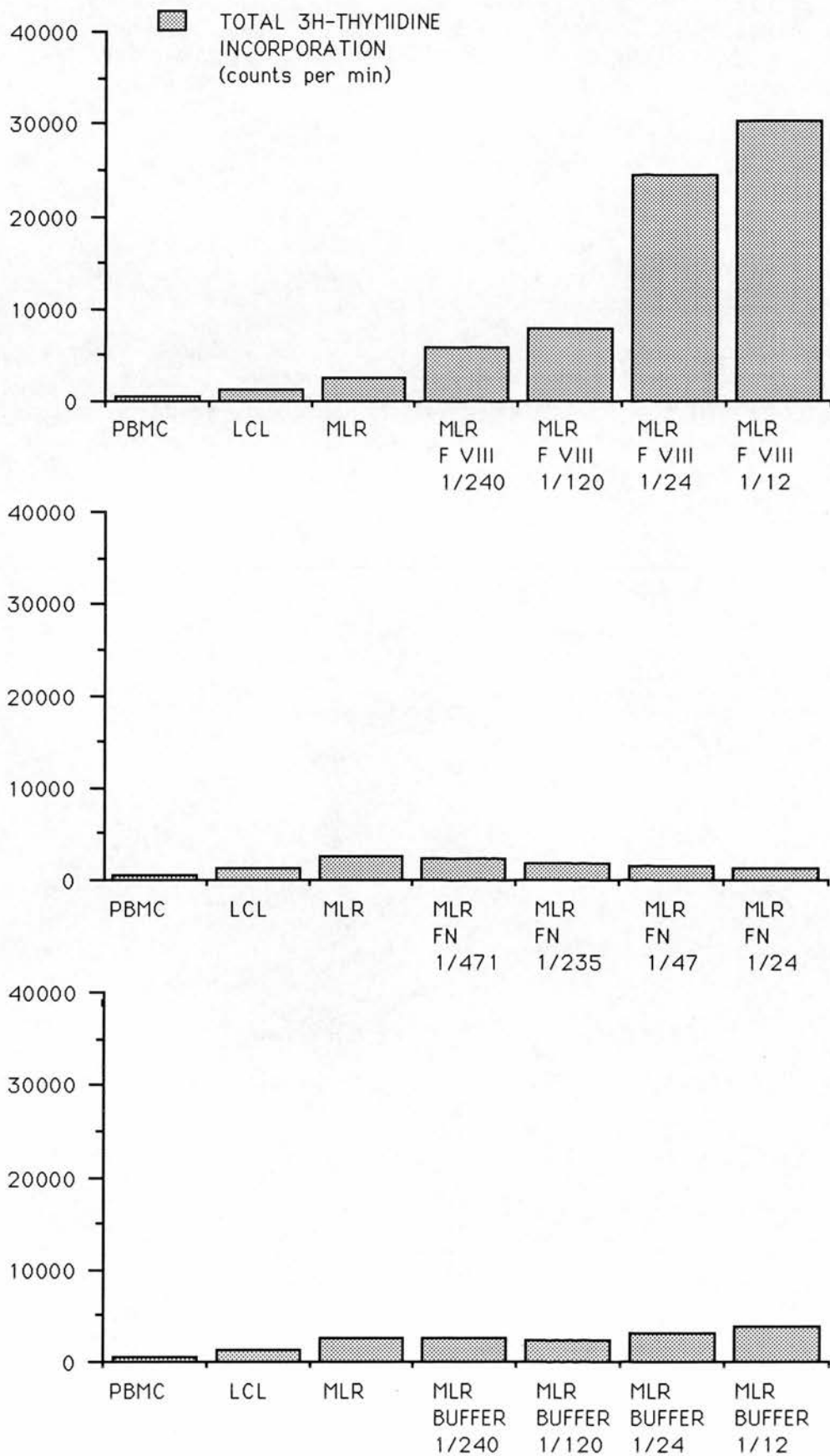


Fig 41

Proliferation in the one way mixed lymphocyte reaction was not enhanced by the presence of fibronectin. Purified human fibronectin was included at a range of concentrations representative of those found in SNBTS blood products.

PREINCUBATION OF SNBTS FACTOR VIII (Z8-92450)
WITH PERIPHERAL BLOOD MONONUCLEAR CELLS OR
IRRADIATED LYMPHOBLASTOID CELLS.

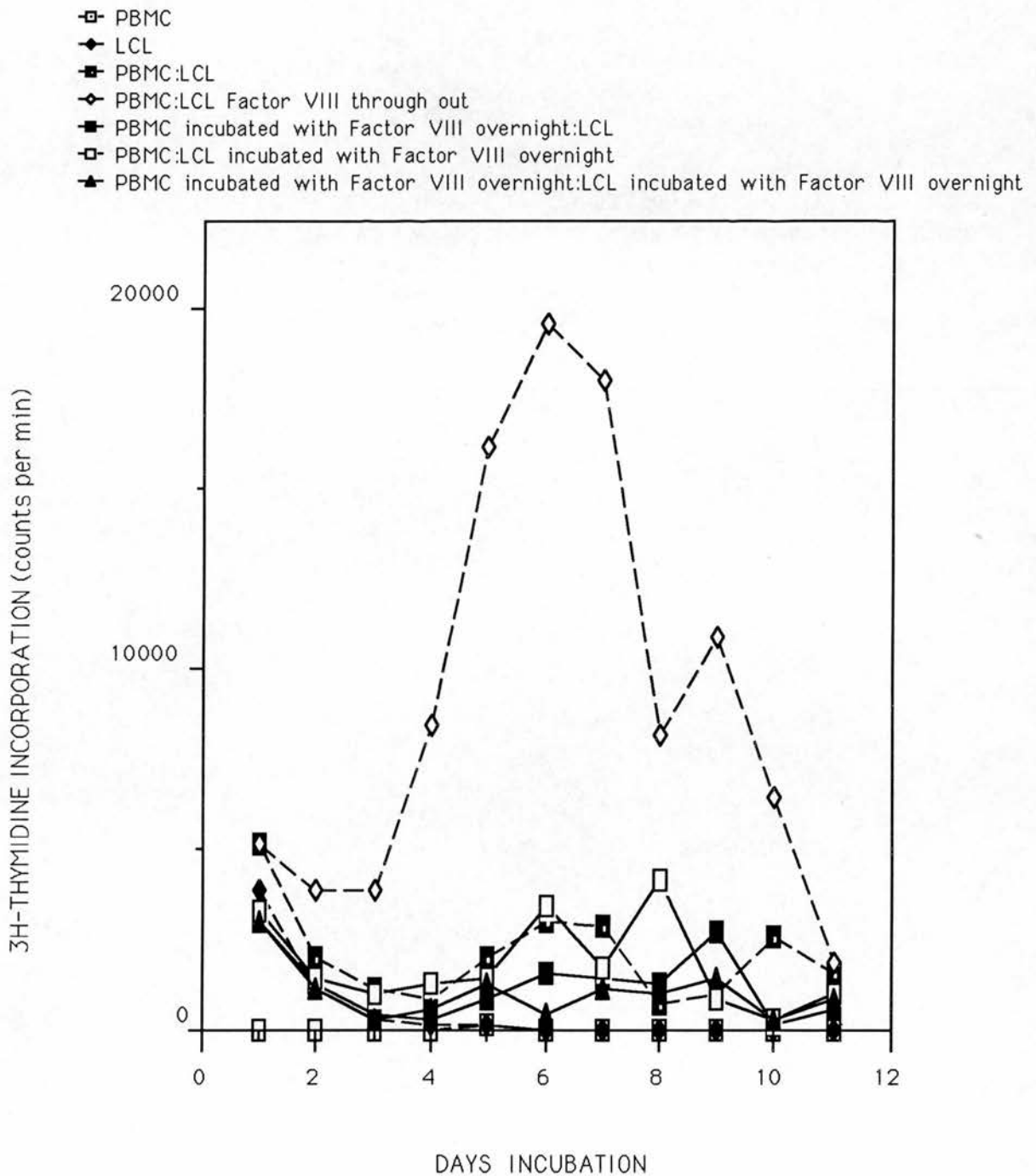


Fig 42

Effect of preincubation of PBMC or irradiated lymphoblastoid cells with factor VIII concentrate prior to inclusion in a mixed lymphocyte reaction. Enhancement of lymphocyte proliferation following stimulation with irradiated lymphoblastoid cells was dependent on the continued presence of SNBTS Z8 concentrate.

mononuclear cells and irradiated lymphoblastoid cells incubated with factor VIII overnight, then washed extensively, reacted weakly when cultured together in a one-way mixed lymphocyte response.

9 Incubation of SNBTS Z8 factor VIII with fresh, fixed, or irradiated lymphoblastoid cells

Peripheral blood mononuclear cells did not respond well to paraformaldehyde fixed allogeneic lymphoblastoid cells, except when factor VIII was present throughout the culture period (Fig 43). The degree of lymphocyte transformation observed was similar to proliferation in response to irradiated lymphoblastoid cells when SNBTS intermediate purity concentrate was present throughout.

Lymphoblastoid cells incubated with factor VIII for four hours prior to fixation did not stimulate peripheral blood mononuclear cells.

10 SDS-PAGE

10.1 Composition of factor VIII concentrates

A range of factor VIII products were electrophoresed in SDS-polyacrylamide gel slabs. Samples were run in both reduced and non reduced forms.

Intermediate purity factor VIII concentrates ran very poorly due to the presence of high molecular weight components (Fig 44, 45).

It is acknowledged that factor VIII concentrates comprise very complex mixtures of plasma proteins at high concentrations. These include albumin, fibrinogen, fibronectin and von Willebrand factor/factor VIII complexes which can have molecular weights of 2 Mega Daltons or more. These mixtures are inherently difficult to handle in biochemical assays, partly because they tend to clot very easily.

Defibrination as described in Materials and Methods section 6.4, probably removed a considerable range of other proteins besides fibrinogen. Following electrophoresis of this material on a 7% polyacrylamide gel, it is evident that many of the components

INCUBATION OF SNBTS FACTOR VIII (Z8-92490) WITH FRESH, FIXED, OR IRRADIATED LYMPHOBLASTOID CELLS

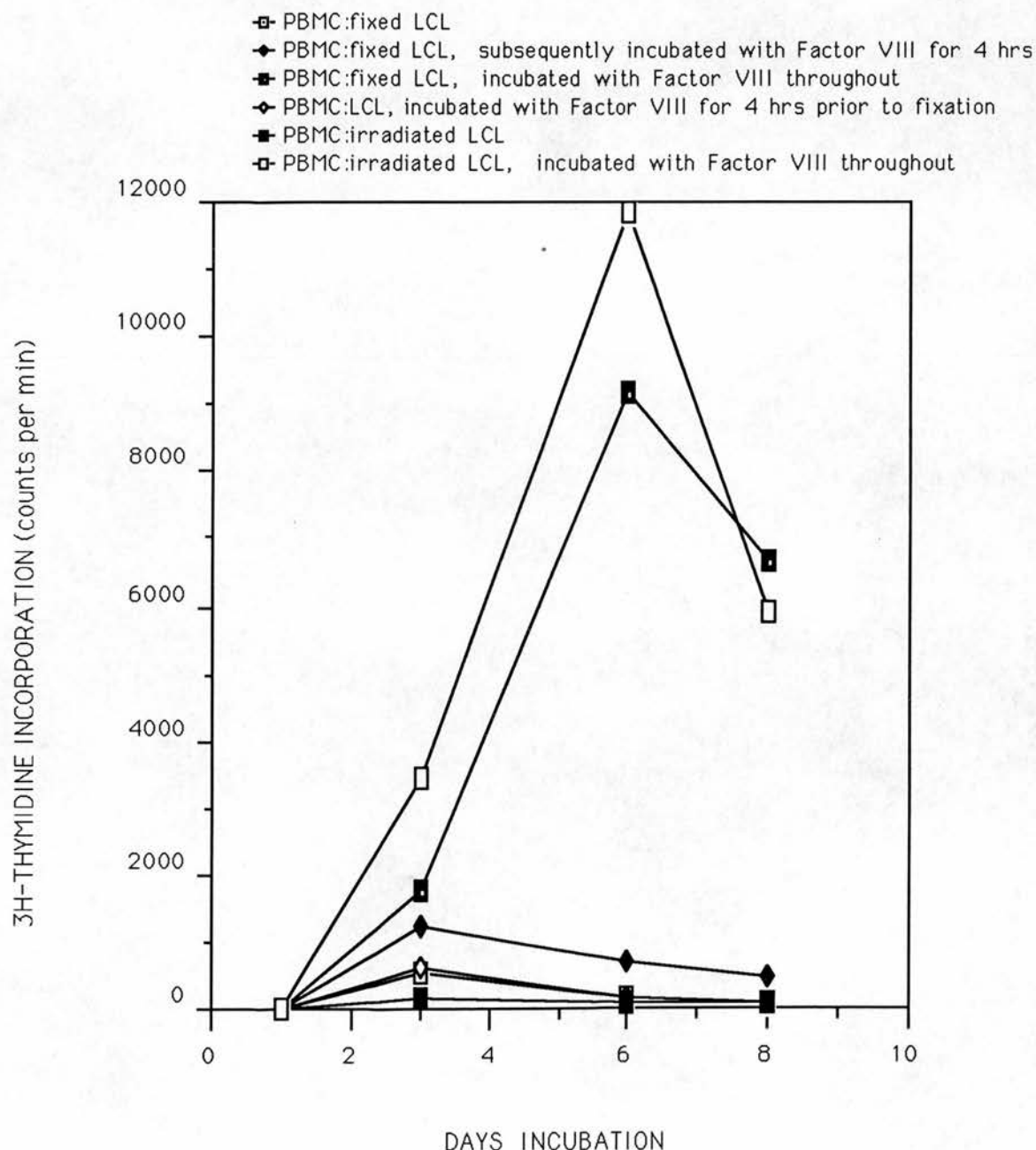
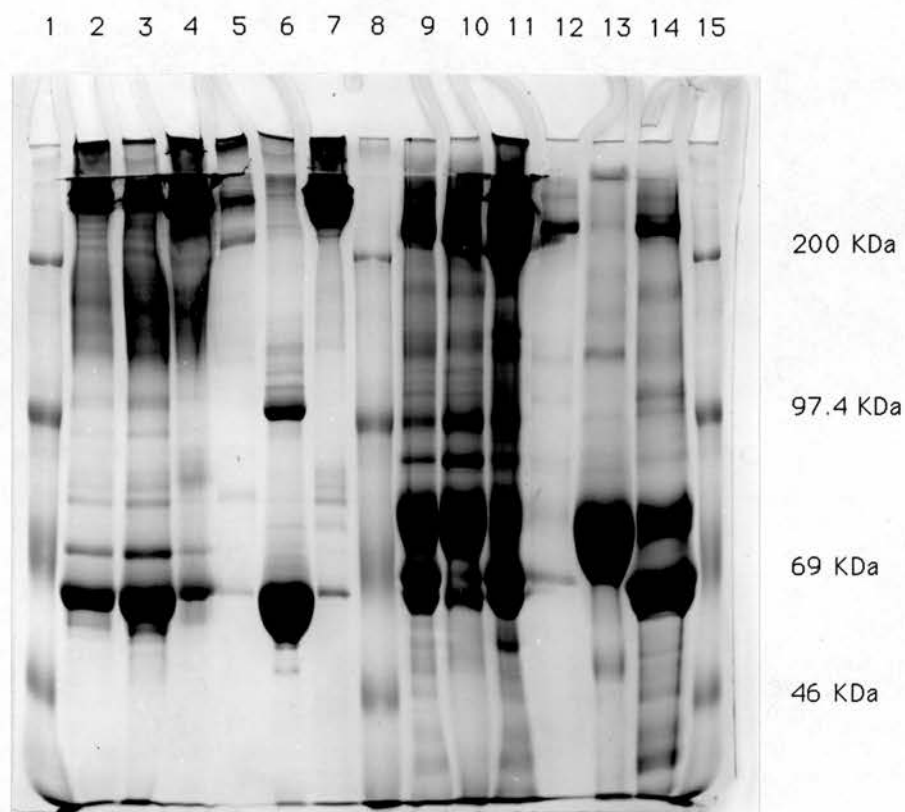
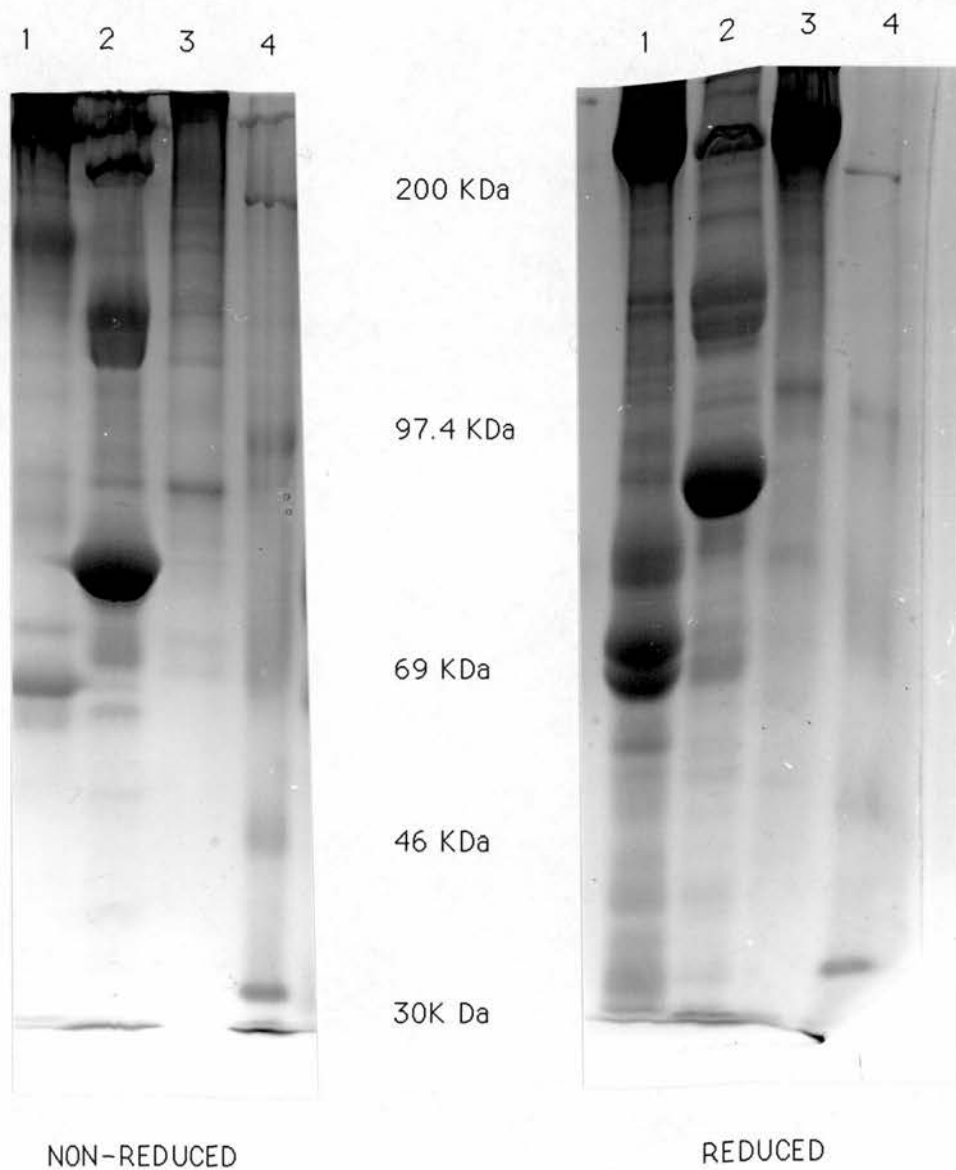


Fig 43
Enhanced lymphocyte proliferation in the one way mixed lymphocyte reaction was dependent upon the continued presence of irradiated or fixed lymphoblastoid cells and SNBTS Z8 factor VIII. Lymphoblastoid cells incubated with factor VIII for four hours before or after fixation did not stimulate lymphocyte proliferation.



LANE NUMBER	SAMPLE	NON REDUCED	REDUCED
1	Marker	+	
2	SNBTS Z8-92340	+	
3	SNBTS Z8-92340 defibrinated	+	
4	SNBTS S8-0006	+	
5	Bio-transfusion	+	
6	Monoclote-P	+	
7	BPL 8Y FHC-0110	+	
8	Marker	+	
9	SNBTS Z8-92340		+
10	SNBTS Z8-92340 defibrinated		+
11	SNBTS S8-0006		+
12	Bio-transfusion		+
13	Monoclote-P		+
14	BPL 8Y FHC-0110		+
15	Marker	+	

Fig 44
SDS polyacrylamide gel of various factor VIII concentrates.



LANE NUMBER	SAMPLE
1	SNBTS S8-0006
2	SNBTS FIX
3	Fibronectin
4	Marker

Fig 45
SDS polyacrylamide gel of SNBTS prototype high purity factor VIII concentrate, S8; SNBTS factor IX concentrate and purified human fibronectin.

remain (Fig 44, lanes 2, 3, 9, 10). There has perhaps been some reduction in the relative quantity of high molecular weight material remaining near the origin and a corresponding increase in the proportion of albumin (the major heavy band of molecular weight approximately 60 K).

The 'higher purity' preparations (SNBTS S8, Monoclone-P, Bio-transfusion and BPL) have, as expected, fewer protein bands than intermediate purity SNBTS Z8. The albumin added as stabilising material in Monoclone-P is very obvious (lanes 6 and 13, Fig 44).

SNBTS factor IX also appears to comprise a complex mixture of proteins (Fig 45, lanes 2). The faint band of approximately 55 K may be factor IX, which has a molecular weight of 55.4 K. (Putman, 1975).

In general, however, the contaminant protein bands observed under both reducing and non-reducing conditions cannot be identified with certainty.

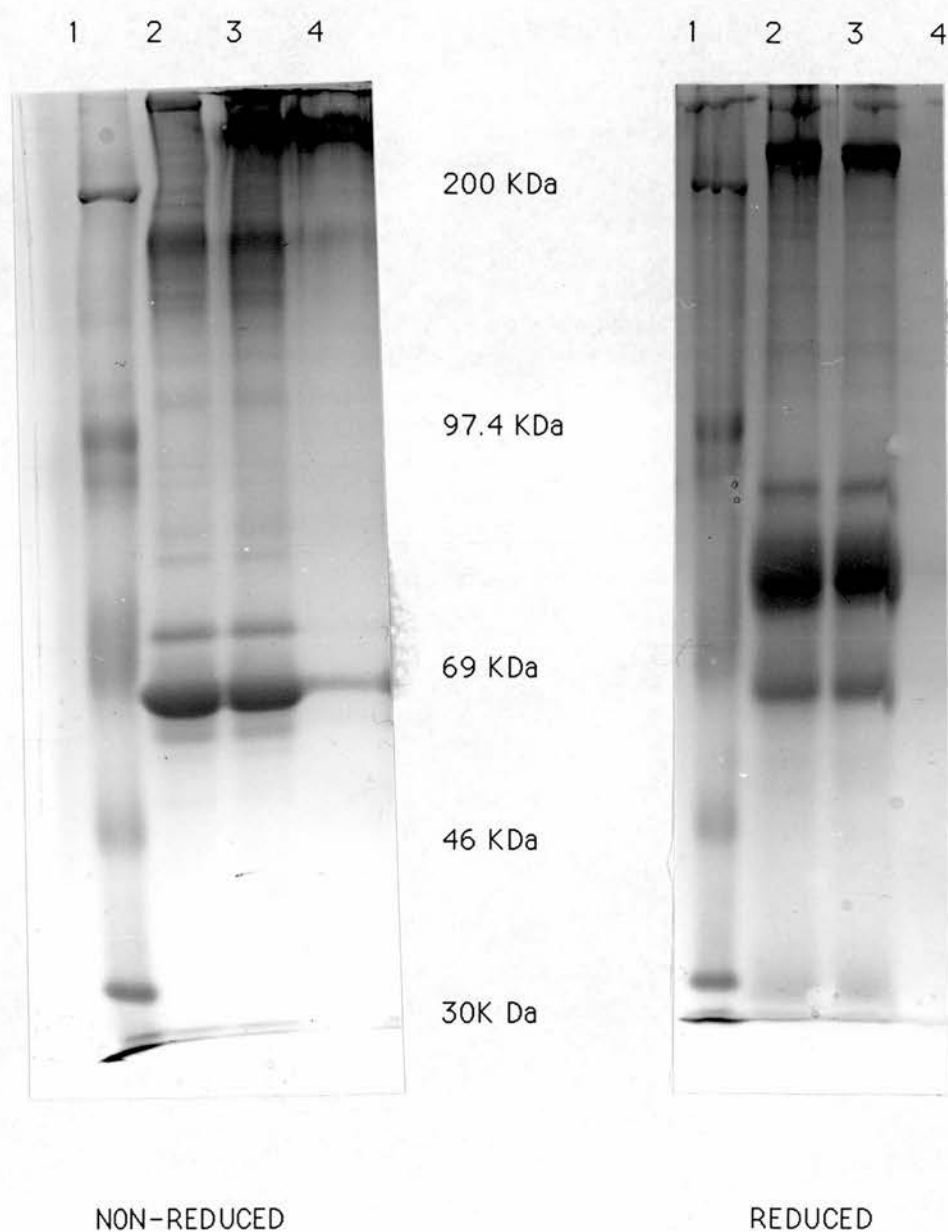
10.2 Electrophoretic profile following filter fractionation

Defibrinated SNBTS factor VIII was fractionated using Amicon Centriprep ultrafiltration devices. The fractions obtained were electrophoresed in a SDS-polyacrylamide gel slab (Fig 46).

Similar protein bands are present in both defibrinated factor VIII and the >100 K fraction of factor VIII (Fig 46, lanes 2 and 3). The presence of protein of <100 K in the >100 K sample does not necessarily mean the fractionation process was unsuccessful. Proteins comprising a number of subunits of <100 K, but with total molecular weight of >100 K would be retained by the >100 K filter fraction, but appear <100 K molecular weight under the denaturing conditions of SDS-PAGE. Very little protein is visible in the <100 K filter fraction (Fig 46, lanes 4). It is apparent that the Centricon cut off filters could not cope with the large concentrations of protein present in this material.

11 Selective protein depletion

Proteins were targeted for removal from SNBTS Z8-92490 factor VIII concentrate by immunomagnetic isolation. The samples obtained were theoretically selectively



LANE NUMBER	SAMPLE
1	Marker
2	SNBTS Z8-92520 defibrinated
3	SNBTS Z8-92520 >100K filter fraction
4	SNBTS Z8-92520 <100K filter fraction

Fig 46
 SDS polyacrylamide gel of defibrinated filtered SNBTS Z8 factor VIII concentrate.

depleted of factor VIII/von Willebrands factor, fibronectin and HLA.DR proteins. When included in lymphocyte proliferation assays all three 'depleted' samples enhanced ^3H -thymidine incorporation in response to irradiated allogeneic lymphoblastoid cells. Control buffer did not enhance proliferation in this assay (Fig 47).

It should be noted that selective protein depletion in this assay was highly improbable due to the presence of inadequate amounts of antibody. For effective protein depletion antibody should have been present in excess. Alternatively proteins could have been removed from factor VIII concentrate following immunoaffinity chromatography using specific antibodies coupled to protein A or protein G sepharose beads.

12 SNBTS factor VIII and HLA.DR expression in lymphoblastoid cells

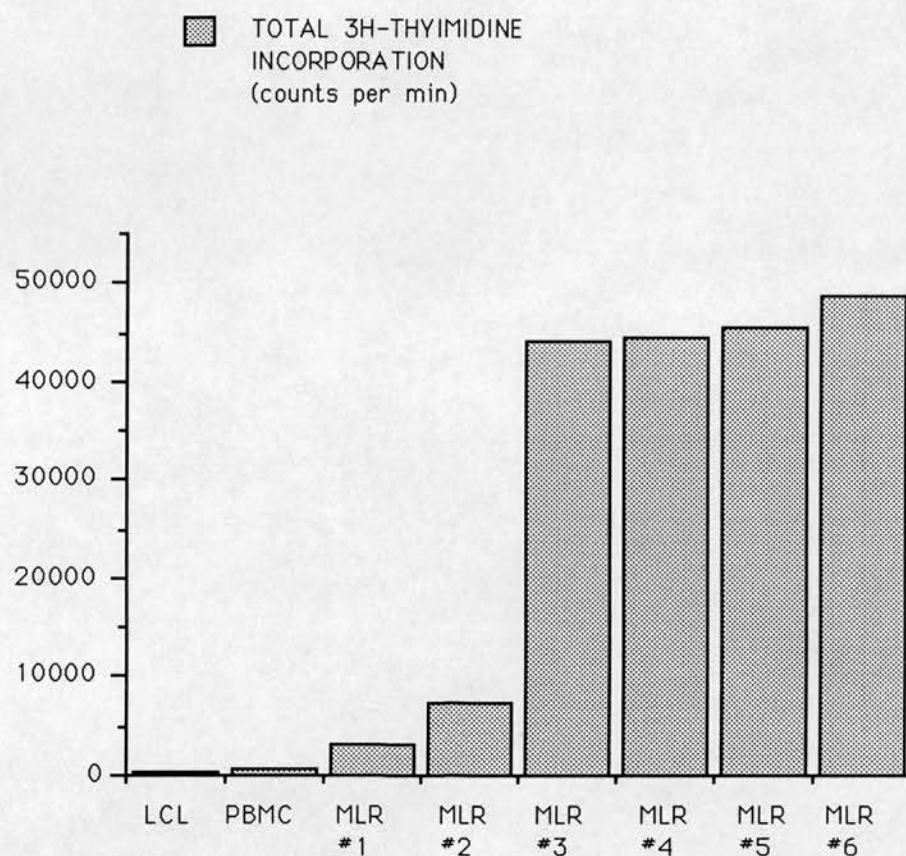
Proliferation in response to allogeneic cells is strongly dependent on HLA.DR recognition. The expression of HLA.DR antigens by the lymphoblastoid cell line RABl₂ was therefore investigated following incubation in the presence or absence of intermediate purity SNBTS factor VIII concentrate.

Factor VIII concentrate did not alter the expression of HLA.DR antigens on irradiated lymphoblastoid cells at the two time points studied (Fig 48).

13 SNBTS factor VIII and cell viability

The effect of Scottish factor VIII on the viability of peripheral blood mononuclear cells and irradiated lymphoblastoid cells was measured by nigrosin dye exclusion.

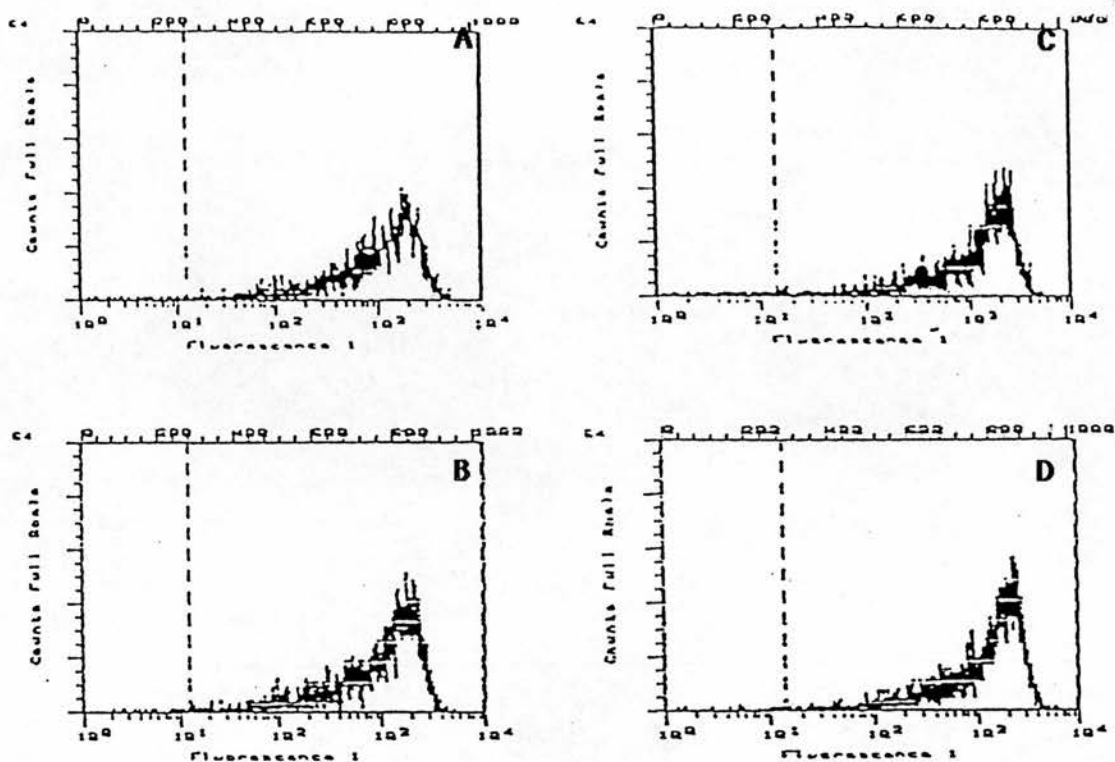
Viability of peripheral blood mononuclear cells and in particular irradiated lymphoblastoid cells was reduced over time. In the presence of factor VIII concentrate cell viability declined less rapidly. The percentage of nigrosin positive cells was significantly reduced in the presence of 1iu/ml SNBTS factor VIII at both 24 and 48 hour time intervals (Table 5).



MLR #1: PBMC:LCL
 MLR #2: PBMC:LCL Buffer 1/12
 MLR #3: PBMC:LCL Factor VIII depleted of HLA.DR 1/12
 MLR #4: PBMC:LCL Factor VIII depleted of Fibronectin 1/12
 MLR #5: PBMC:LCL Factor VIII depleted of Factor VIII/von Willebrands Factor 1/12
 MLR #6: PBMC:LCL Factor VIII 1/12 (1iu/ml)

Fig 47

Several protein constituents of factor VIII concentrate were targeted for depletion using immunomagnetic isolation. Depleted samples were assayed for co-mitogenic activity in the mixed lymphocyte reaction.



The expression of HLA-DR on the human B cell line RAB12 following incubation in the presence or absence of 2iu/ml SNBTS Z8 factor VIII concentrate.

- A RAB12 incubated in culture medium, 24 hours
- B RAB12 incubated in culture medium supplemented with SNBTS factor VIII, 24 hours
- C RAB12 incubated in culture medium, 48 hours
- D RAB12 incubated in culture medium supplemented with SNBTS factor VIII, 48 hours

Fig 48
 SNBTS factor VIII concentrate did not alter the expression of HLA-DR antigens on irradiated lymphoblastoid cells at two time points studied.

% CELLS NIGROSIN POSITIVE

	0 HOURS	24 HOURS	48 HOURS
PBMC	1.5	10	19.5
LCL	15	48.5	62
PBMC incubated with FACTOR VIII	1.5	3.5 [†]	2.2 [†]
LCL incubated with FACTOR VIII	15	16 [†]	21 [†]

[†] $P < 0.01$

Table 5
 Viability of PBMC and LCL in the presence or absence of 1 iu/ml SNBTS Z8 factor VIII.

14 Mixed lymphocyte reactions and culture media

Lymphocyte proliferation in response to irradiated allogeneic lymphoblastoid cells was fairly limited when cells were cultured in medium supplemented with 5% heat-inactivated foetal calf serum. In contrast, cells grown in medium containing 10% autologous plasma responded well to allogeneic stimulator cells.

SNBTS intermediate purity factor VIII did not further enhance lymphocyte proliferation in a one-way mixed lymphocyte reaction performed in culture medium supplemented with 10% autologous plasma (Fig 49). Similarly, control buffer did not affect ^3H -thymidine incorporation in response to irradiated allogeneic cells when culture medium contained autologous plasma (Fig 50).

15 Lymphocyte proliferation assays and HIV infection

15.1 PHA stimulation

Peripheral blood mononuclear cells were infected with HIV prior to stimulation with PHA and incubated in the presence or absence of SNBTS intermediate purity factor VIII concentrate.

Factor VIII inhibited ^3H -thymidine incorporation in response to PHA in a dose-dependent manner. Virus replication, as assessed by HIV p24 antigen production, was reduced in the presence of factor VIII concentrate (Fig 51).

Lymphocyte proliferation curves for mock infected peripheral blood mononuclear cells were similar to those for HIV-infected lymphocytes. Mock infected control cultures were consistently negative for HIV p24 antigen.

15.2 One-way mixed lymphocyte reactions

Peripheral blood mononuclear cells were infected with HIV prior to inclusion in a weak one-way mixed lymphocyte reaction and incubated in the presence or absence of SNBTS intermediate purity factor VIII.

^3H -thymidine incorporation was significantly enhanced in the presence of SNBTS Z8 concentrate. HIV p24 antigen production increased in parallel with enhanced

SNBTS Z8-02870
AUTOLOGOUS PLASMA

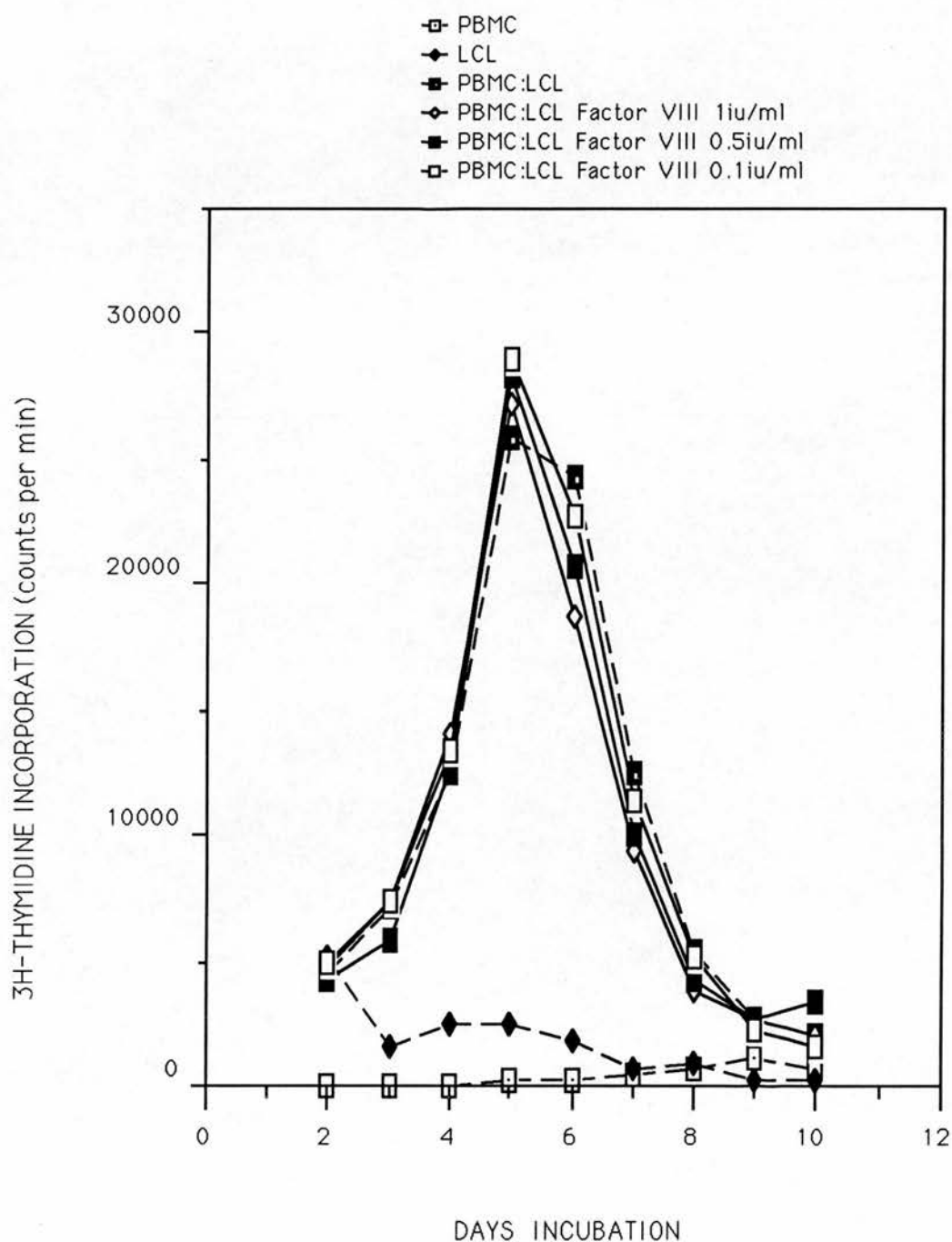


Fig 49
 SNBTS Z8 factor VIII did not effect lymphocyte proliferation in response to irradiated allogeneic stimulator cells when cultured in growth medium supplemented with 10% autologous plasma.

SNBTS BUFFER **AUTOLOGOUS PLASMA**

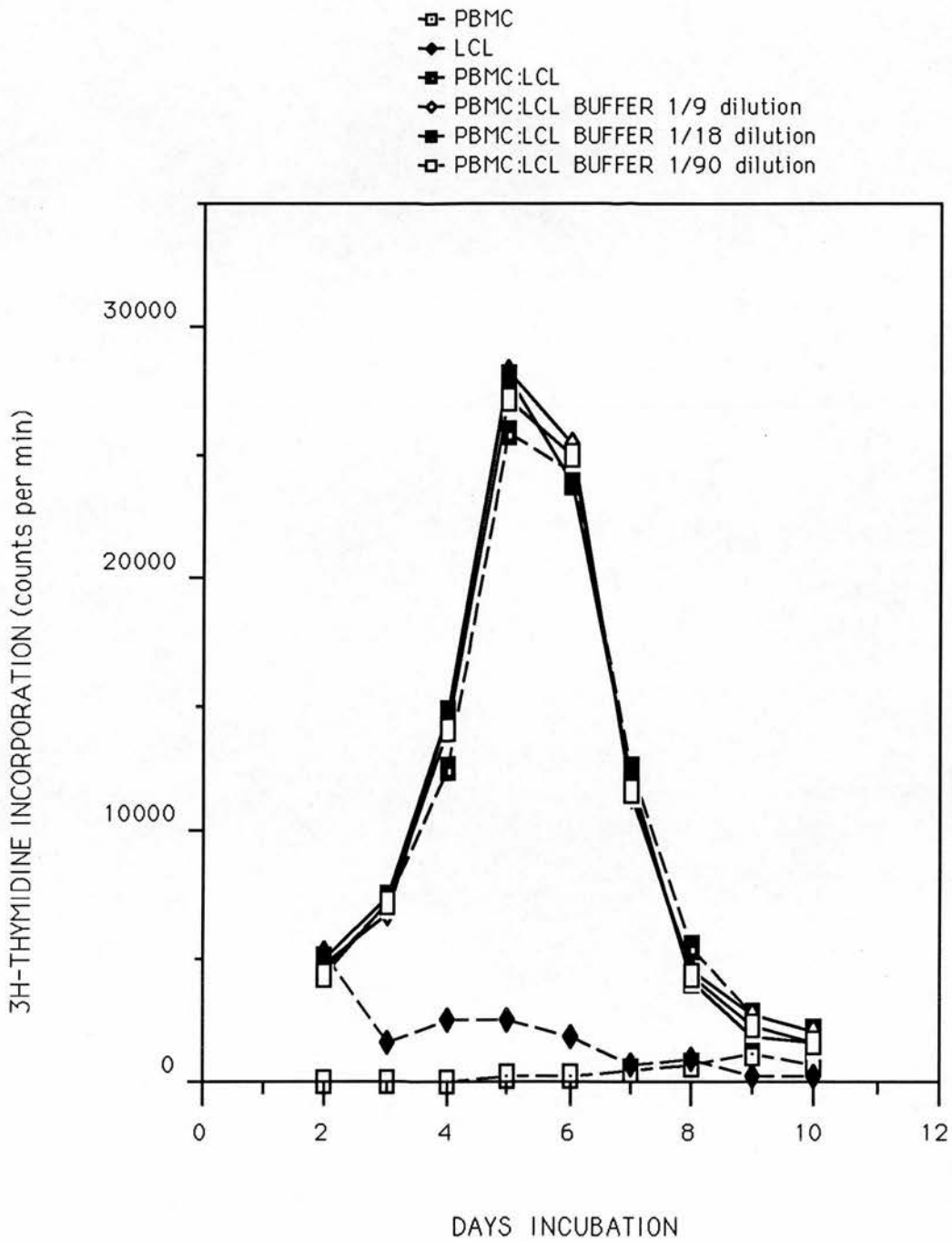


Fig 50
 SNBTS control buffer did not effect lymphocyte proliferation in response to irradiated allogeneic stimulator cells when cultured in growth medium supplemented with 10% autologous plasma.

SNBTS Z8-92450
HIV-infected PBMC

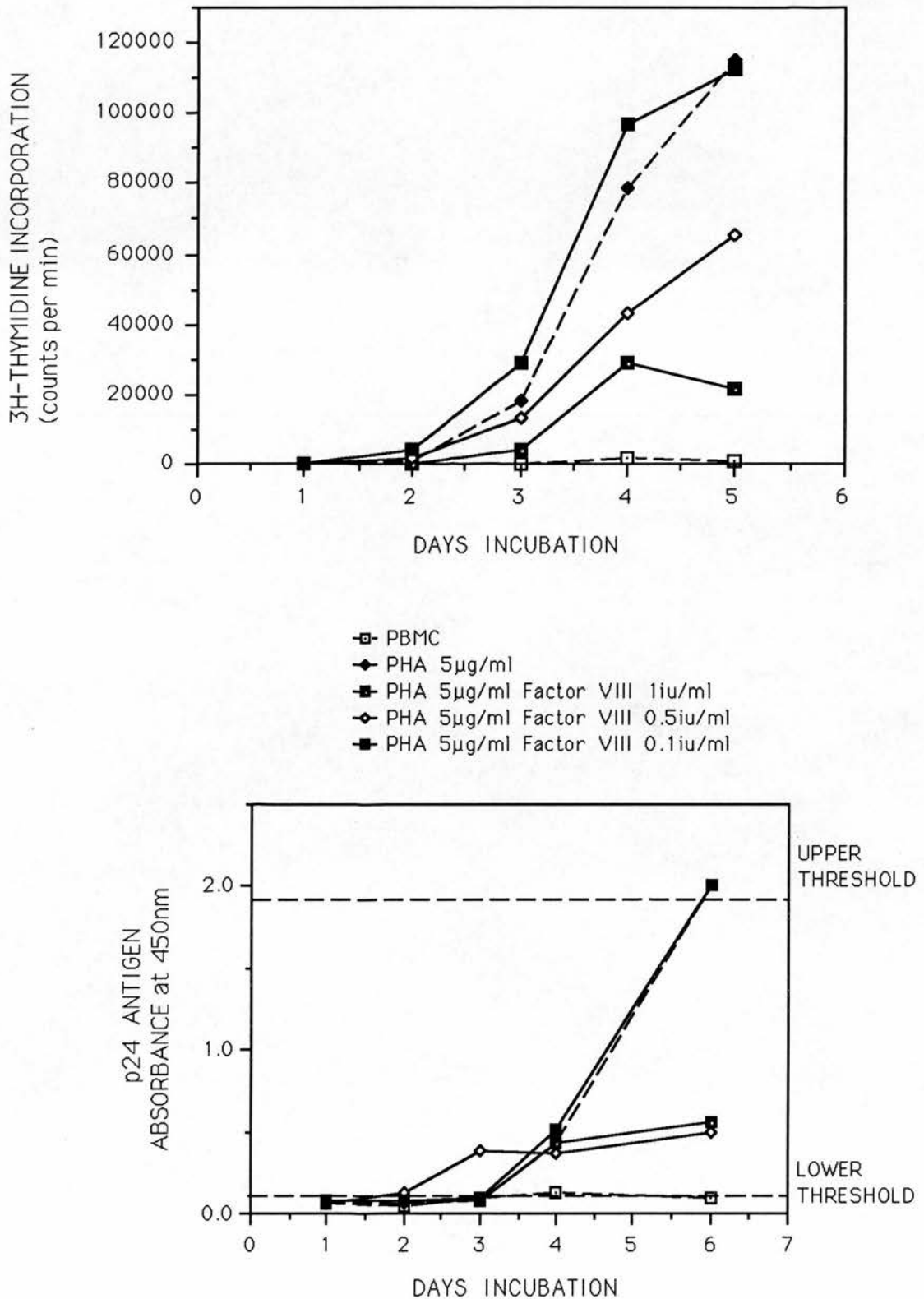


Fig 51
Inhibition of lymphocyte proliferation and HIV virus proliferation as measured by 3H-thymidine incorporation and p24 antigen production respectively in the presence or absence of SNBTS Z8 factor VIII. Lymphocytes were stimulated with 5 µg/ml PHA. Total protein included per assay ranged from 0.26 mg/ml to 2.56 mg/ml.

lymphocyte proliferation (Fig 52).

Peripheral blood mononuclear cells did not respond to factor VIII in the absence of irradiated allogeneic stimulator cells. Similarly, p24 antigen was not detected in cultures containing peripheral blood mononuclear cells and factor VIII alone.

Mock infected lymphocytes showed enhanced ^3H -thymidine incorporation in a weak one-way mixed lymphocyte reaction in the presence of factor VIII. Mock infected control cultures were negative for p24 antigen throughout.

16 Mixed lymphocyte reactions in haemophilia patients

16.1 HIV antibody negative

Two HIV antibody negative haemophilia patients responded well to irradiated allogeneic stimulator cells, when cultured in medium containing 10% autologous plasma. Factor VIII did not enhance proliferation in the one-way mixed lymphocyte reaction over a range of doses (Fig 53, 54).

Neither patient responded to factor VIII concentrate alone.

16.2 HIV antibody positive

Peripheral blood mononuclear cells from two HIV antibody positive haemophilia patients were stimulated with irradiated allogeneic lymphoblastoid cells in a one-way mixed lymphocyte reaction.

Cells from patient (3) were cultured in medium containing 5% pooled (AB) human serum, while those from patient (4) were maintained in medium supplemented with 10% autologous heat-inactivated plasma.

Patient lymphocytes did not respond well in one-way mixed lymphocyte cultures.

Although some proliferation was evident on day two or three of culture, ^3H -thymidine incorporation decreased with time. On the whole ^3H -thymidine incorporation was very low.

SNBTS factor VIII appeared to enhance proliferation in response to stimulator cells to some extent (Fig 55, 57). Control buffer or Monoclone-P factor VIII concentrate did not have this effect (Fig 56, 58, 59).

SNBTS Z8-92450
HIV-infected PBMC

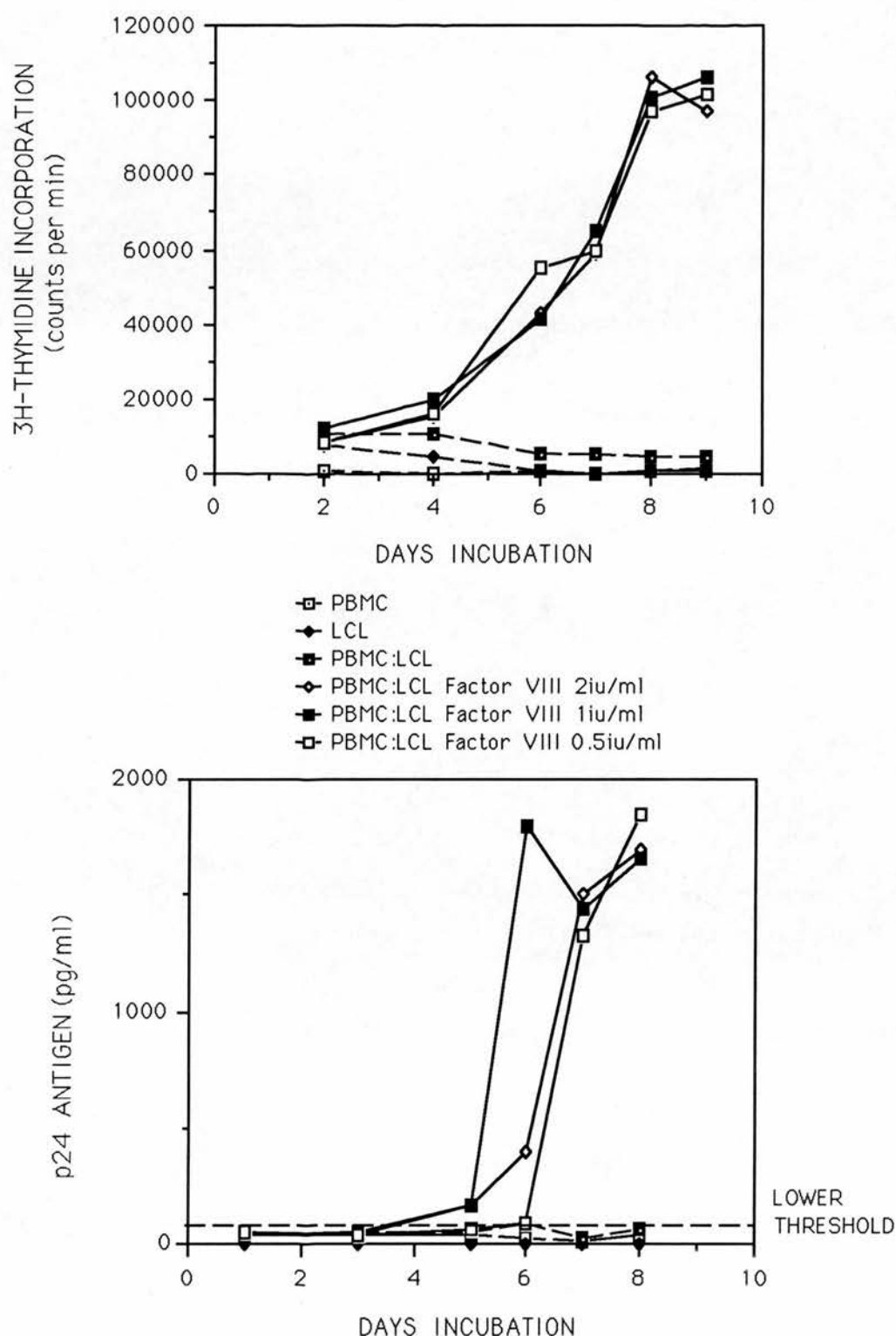


Fig 52
Enhanced lymphocyte proliferation and HIV replication as measured by 3H-thymidine incorporation and p24 antigen production respectively in the one way mixed lymphocyte reaction. Lymphocytes were stimulated with irradiated allogeneic stimulator cells. Total protein included per assay ranged from 1.28 mg/ml to 5.11 mg/ml.

SNBTS Z8-02970

AUTOLOGOUS PLASMA

HIV-antibody negative haemophilia patient (1)

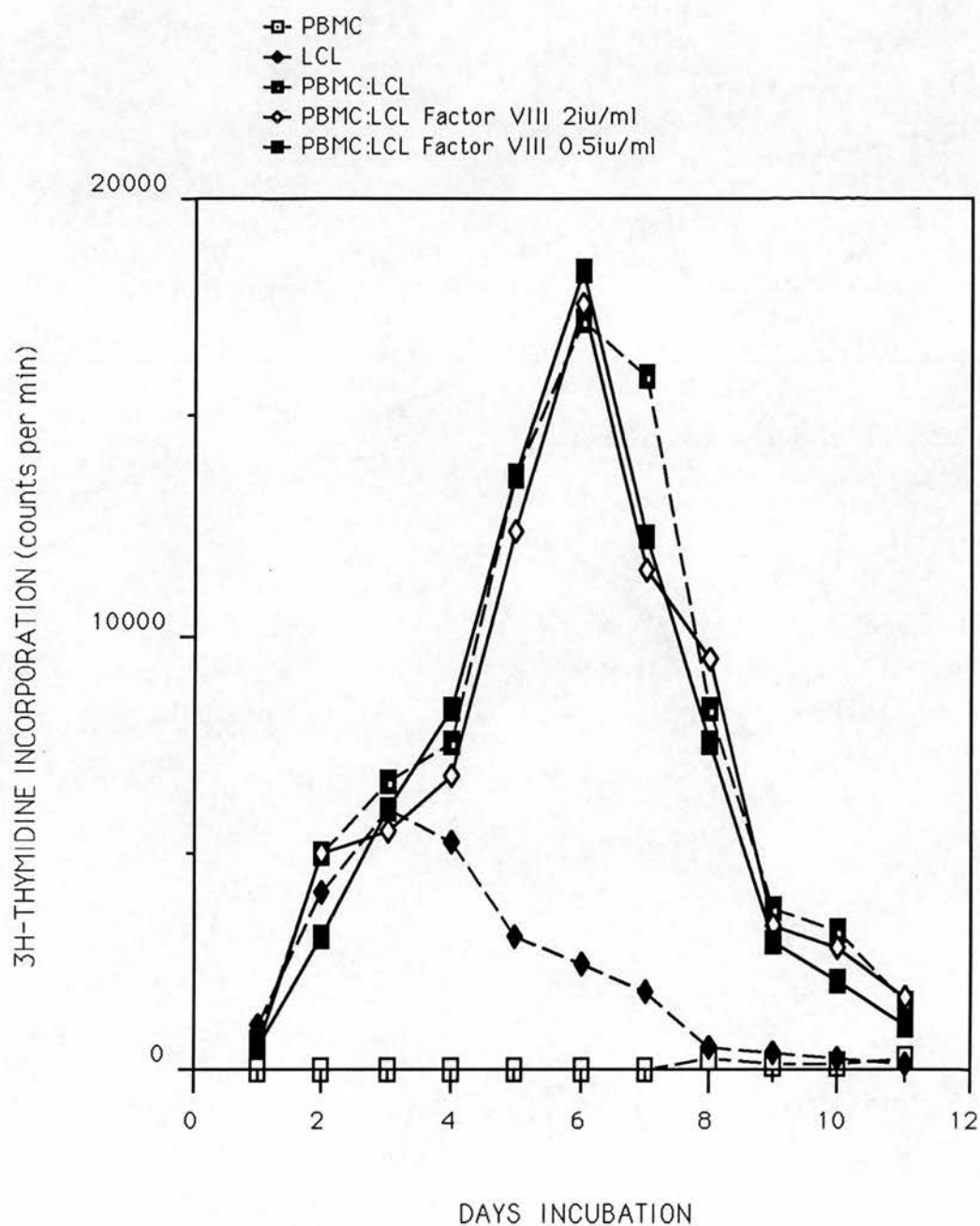


Fig 53

SNBTS Z8 factor VIII did not effect lymphocyte proliferation in a mixed lymphocyte reaction including lymphocytes from a HIV sero-negative haemophilia patient. The proliferation assay was performed in growth medium containing 10% autologous plasma.

SNBTS Z8-02870
AUTOLOGOUS PLASMA
HIV-antibody negative haemophilia patient (2)

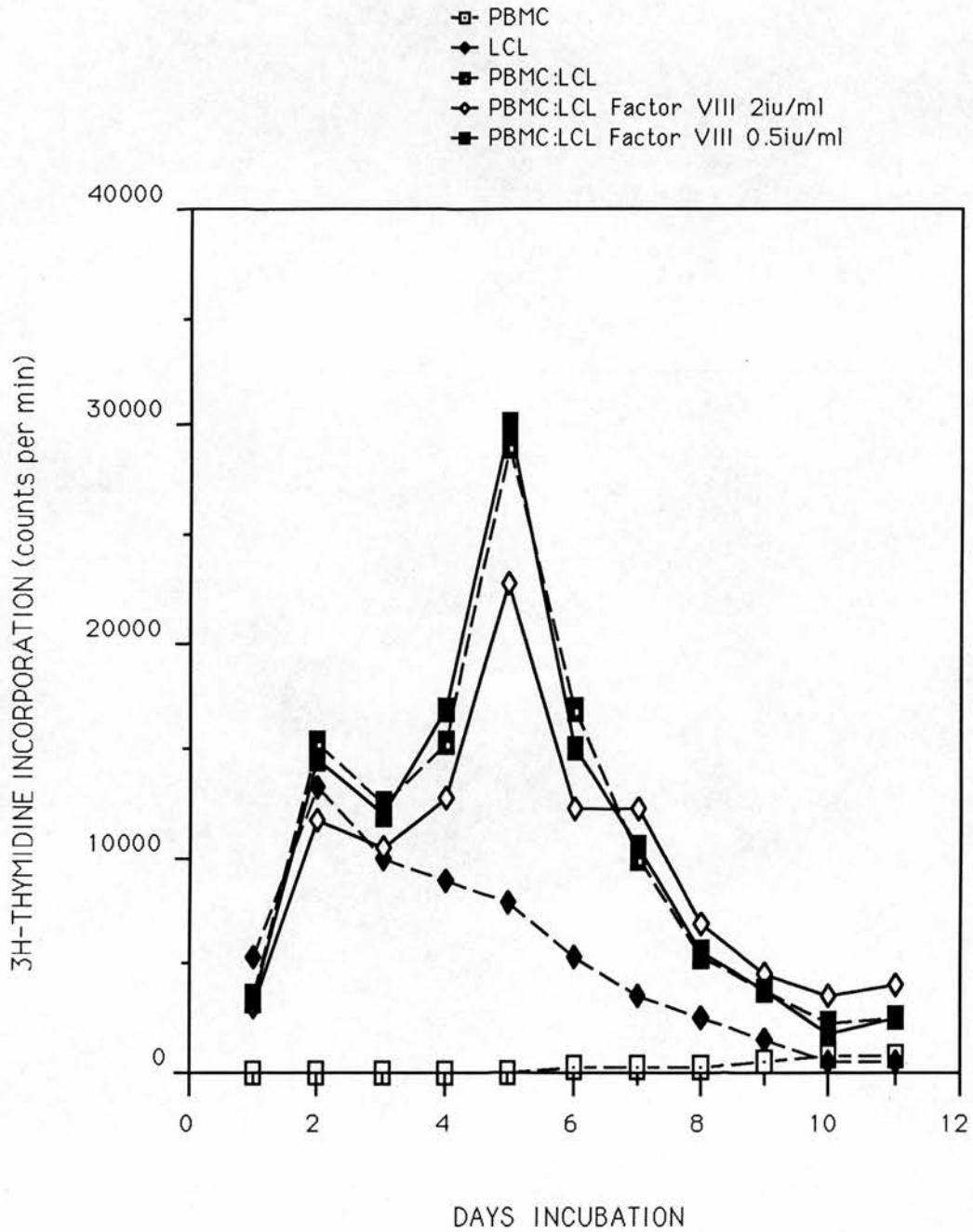


Fig 54
SNBTS Z8 factor VIII did not effect lymphocyte proliferation in a mixed lymphocyte reaction including lymphocytes from a HIV sero-negative haemophilia patient. The proliferation assay was performed in growth medium containing 10% autologous plasma.

SNBTS Z8-92520

HIV-antibody positive haemophilia patient (3)
POOLED (AB) HUMAN SERUM

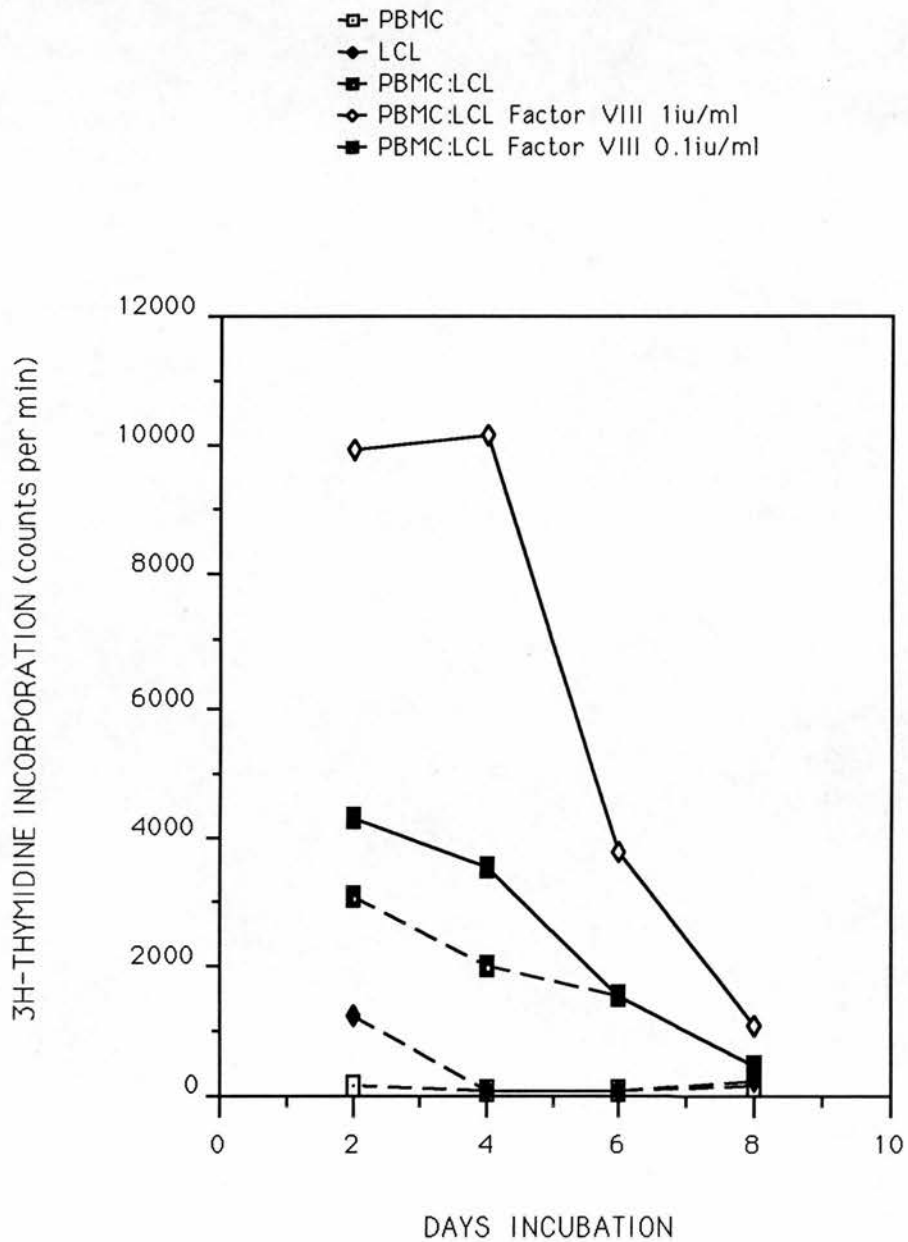


Fig 55

Effect of SNBTS Z8 factor VIII concentrate on lymphocyte proliferation in the mixed lymphocyte reaction in a HIV sero-positive haemophilia patient. The proliferation assay was performed in growth medium containing 5% pooled (AB) human serum. Total protein included per assay ranged from 0.22 mg/ml to 2.18 mg/ml.

SNBTS BUFFER

HIV-antibody positive haemophilia patient (3)
POOLED (AB) HUMAN SERUM

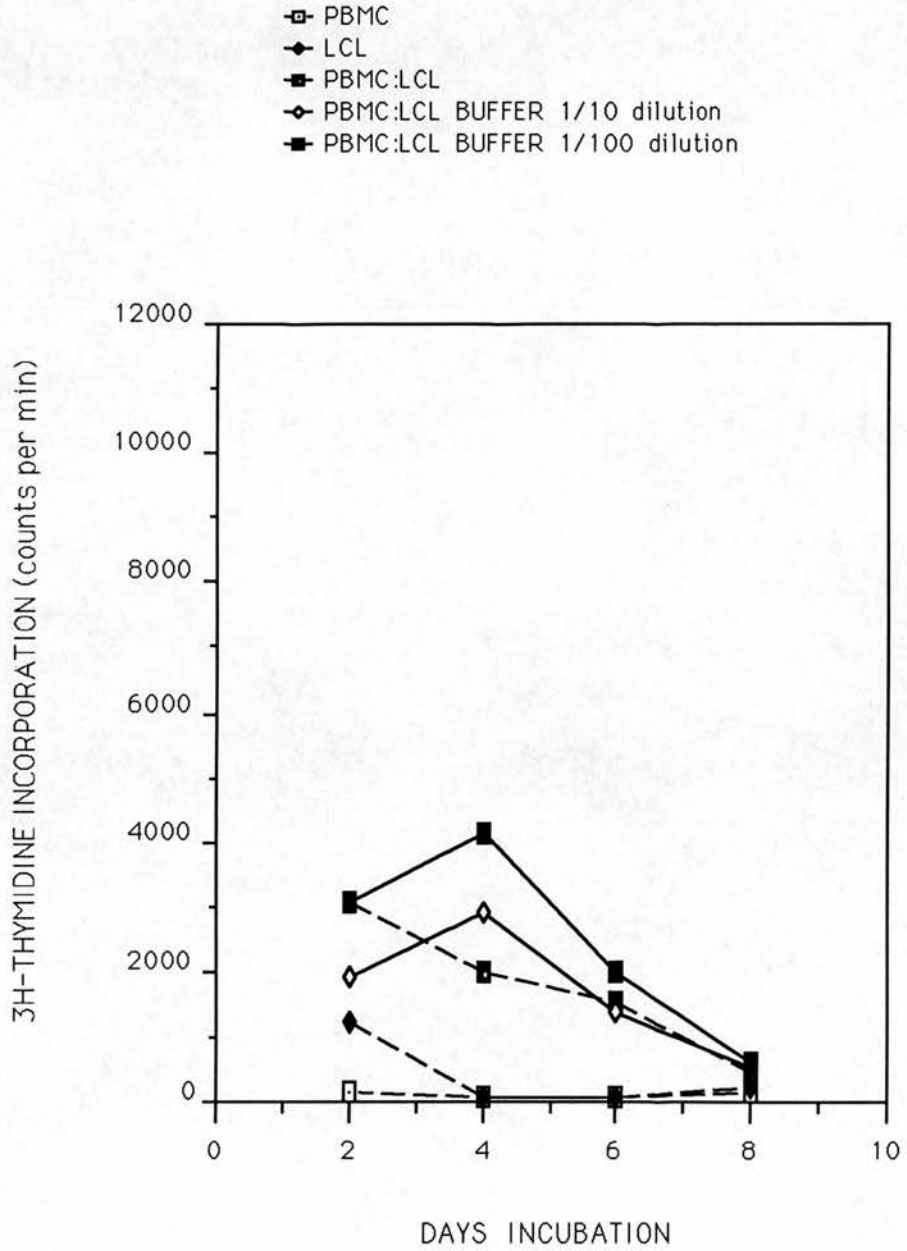


Fig 56

Effect of SNBTS control buffer on lymphocyte proliferation in the mixed lymphocyte reaction in a HIV sero-positive haemophilia patient. The proliferation assay was performed in growth medium containing 5% pooled (AB) human serum.

SNBTS Z8-92520

HIV-antibody positive haemophilia patient (4)
HEAT-INACTIVATED AUTOLOGOUS PLASMA

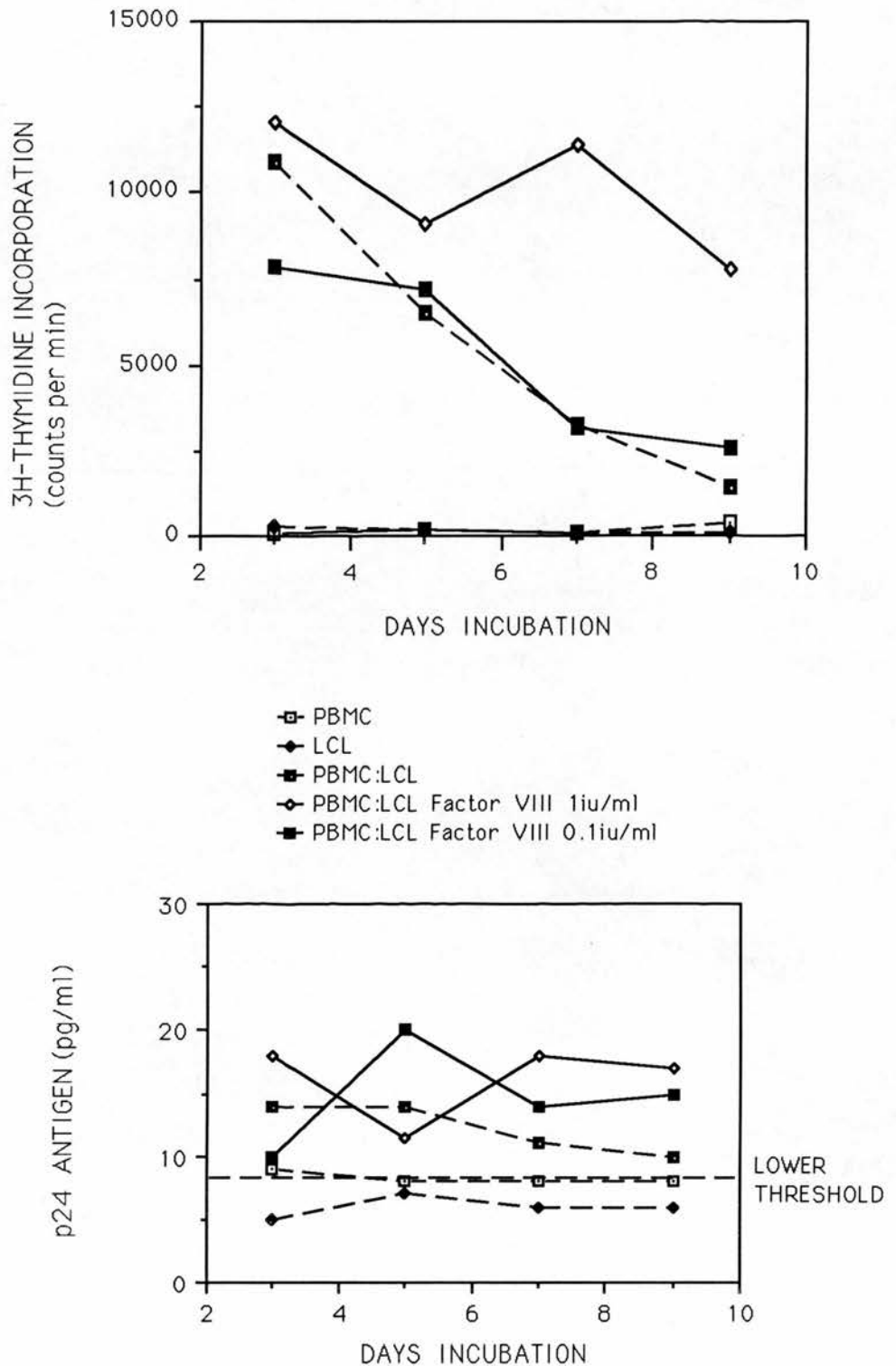


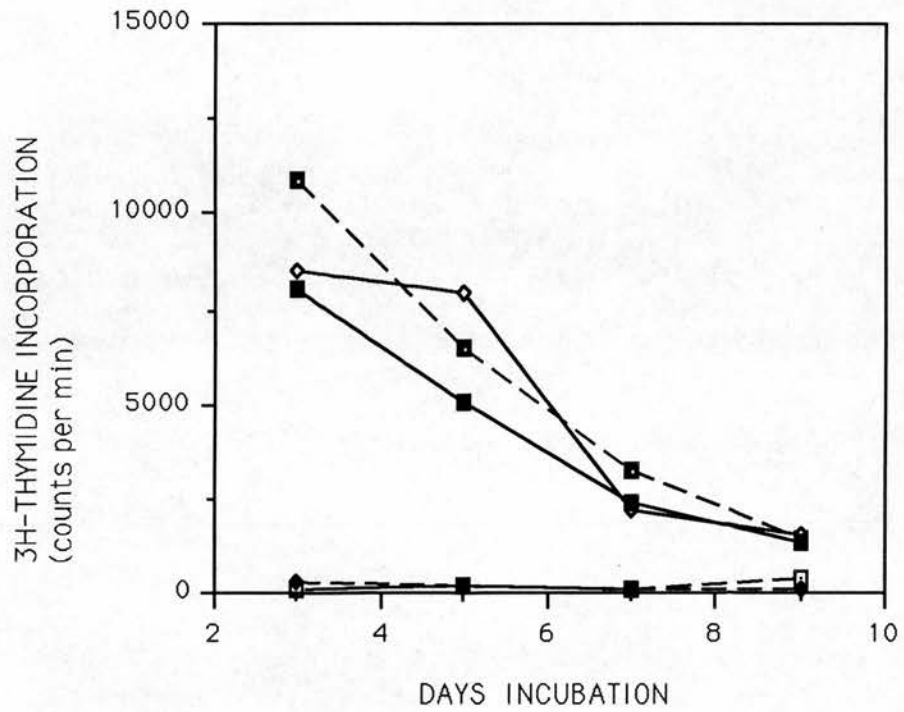
Fig 57

Lymphocyte proliferation and HIV replication as measured by 3H-thymidine incorporation and p24 antigen production respectively in the mixed lymphocyte reaction in a HIV antibody positive haemophilia patient. Cultures were performed in medium containing 10% autologous heat-inactivated plasma in the presence or absence of SNBTS Z8 factor VIII. Total protein included per assay ranged from 0.22 mg/ml to 2.18 mg/ml.

SNBTS BUFFER

HIV-antibody positive haemophilia patient (4)

HEAT-INACTIVATED AUTOLOGOUS PLASMA



- PBMC
- ◆ LCL
- PBMC:LCL
- ◇ PBMC:LCL Buffer 1/10 dilution
- PBMC:LCL Buffer 1/100 dilution

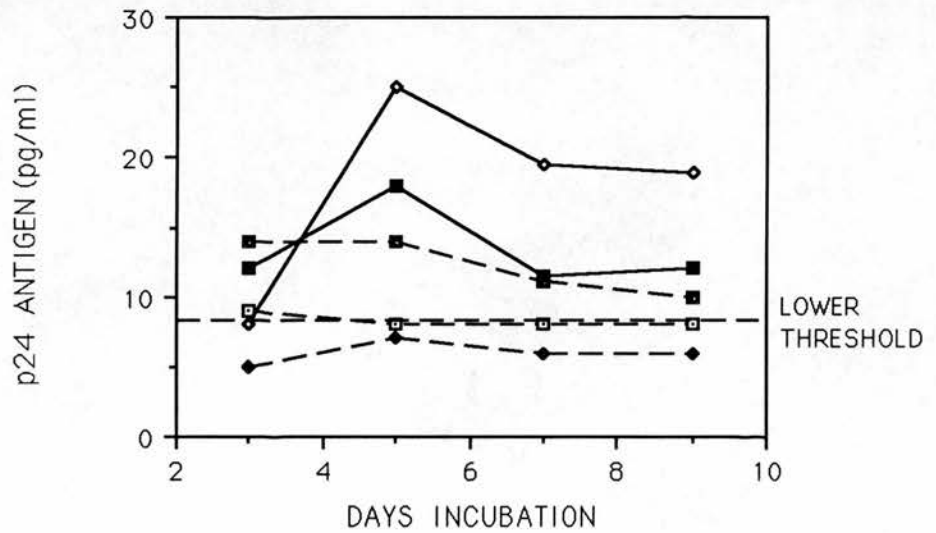


Fig 58

Lymphocyte proliferation and HIV replication as measured by 3H-thymidine incorporation and p24 antigen production respectively in the mixed lymphocyte reaction in a HIV antibody positive haemophilia patient. Cultures were performed in medium containing 10% autologous heat-inactivated plasma in the presence or absence of SNBTS control buffer.

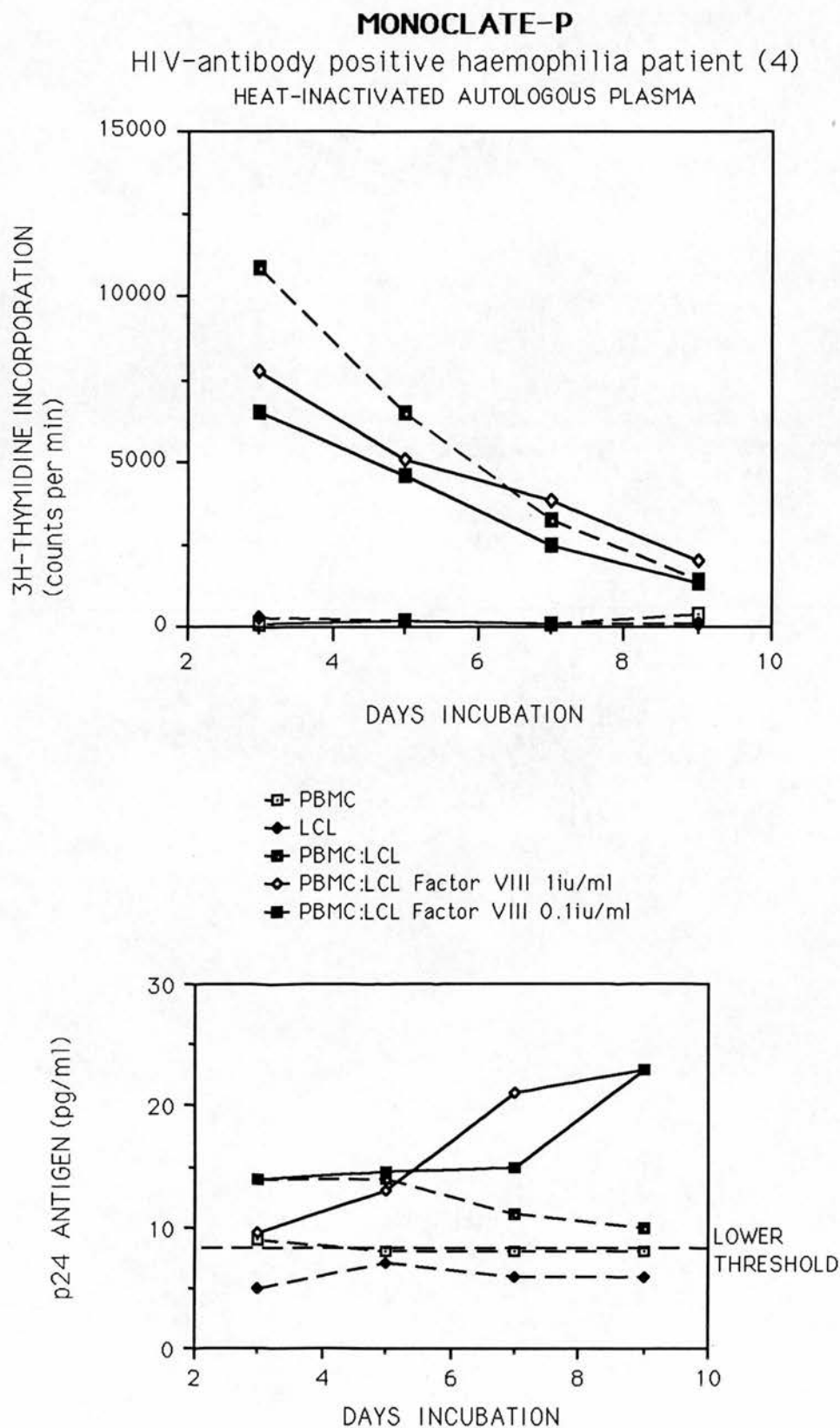


Fig 59

Lymphocyte proliferation and HIV replication as measured by ^3H -thymidine incorporation and p24 antigen production respectively in the mixed lymphocyte reaction in a HIV antibody positive haemophilia patient. Cultures were performed in medium containing 10% autologous heat-inactivated plasma in the presence or absence of monoclonal antibody purified Monoclote-P factor VIII concentrate. Total protein included per assay ranged from 7.5×10^{-3} to 7.5×10^{-2} .

HIV p24 antigen was not detected in cell culture supernatants from patient (3). In patient (4), p24 antigen was detected in cultures stimulated with irradiated lymphoblastoid cells in the presence or absence of SNBTS Z8, control buffer and Monoclone-P (Fig 57, 58, 59).

There did not appear to be any correlation between lymphocyte proliferation and HIV p24 antigen production.

Neither patient responded to factor VIII alone, either by lymphocyte proliferation or by p24 antigen production.

The effects of different factor concentrates on various lymphocyte proliferation assays are summarised in Table 6.

TABLE 6

Summary of the effects of different factor VIII concentrates in various proliferation assay systems.

KEY	
+++	ENHANCED
---	INHIBITED
+/-	NO EFFECT
++/--	ENHANCED AND INHIBITED (concentration dependent)
-	INHIBITED AT HIGH CONCENTRATION ONLY
+	ENHANCED AT HIGH CONCENTRATION ONLY
ND	NOT DETERMINED

FACTOR CONCENTRATE	GROWTH MEDIUM SUPPLEMENT	PHA STIMULATION					TETANUS/PWM		MLR
		PROLIFERATION	CD 25 EXPRESSION	CD 71 EXPRESSION	HLA-DR EXPRESSION	IL2 PRODUCTION	PROLIFERATION	PROLIFERATION	
SNBTS Z8	FCS	---	---	---	+/-	---	ND	+++	
	AUTOLOGOUS PLASMA	ND	ND	ND	ND	ND	ND	+/-	
SNBTS Z8-6026	FCS	++/--	ND	ND	ND	ND	++/--	ND	
SNBTS S8	FCS	ND	---	---	+/-	ND	ND	+++	
SNBTS FACTOR IX	FCS	---	ND	ND	ND	ND	ND	+	
SNBTS CONTROL BUFFER	FCS	DELAYED	---	---	+/-	ND	ND	+/-	
	AUTOLOGOUS PLASMA	ND	ND	ND	ND	ND	ND	+/-	
BIO PRODUCTS LABORATORY FACTOR VIII	FCS	---	ND	ND	ND	ND	ND	+++	
PROFILATE	FCS	-	ND	ND	ND	ND	ND	+/-	
OCTA-PHARMA V.I. 250	FCS	+/-	ND	ND	ND	ND	ND	+/-	
MONOCLATE	FCS	+/-	ND	ND	ND	ND	ND	ND	
HEMOFIL-M	FCS	+/-	ND	ND	ND	ND	ND	+/-	
MONOCLATE-P	FCS	+/-	+/-	+/-	+/-	ND	ND	+/-	
BIO-TRANSFUSION	FCS	+/-	ND	ND	ND	ND	ND	+/-	

Table 6. Summary of experimental results.

DISCUSSION

In agreement with a number of published studies (Froebel et al, 1983, Lederman et al, 1986, Hay et al, 1990) I have demonstrated that intermediate purity factor VIII concentrates can modulate immune reactions *in vitro*.

1 Effects of factor VIII concentrate on lymphocyte proliferation following stimulation with phytohaemagglutinin

Lymphocyte proliferation in response to the non-specific T cell mitogen PHA was severely depressed in the presence of high levels of SNBTS Z8 factor VIII concentrate. McDonald et al (1985) reported that inhibition of lymphocyte transformation by Scottish factor VIII could be reproduced when buffers used in the manufacture of factor VIII were substituted for the concentrates themselves. The authors proposed that citrate present in buffer and concentrate inhibited lymphocyte transformation by reducing the availability of calcium ions required for lymphocyte activation *in vitro*. However, in that study ^3H -thymidine incorporation was assessed at one time point only, 50 hours after mitogen stimulation.

My own studies have also shown that ^3H -thymidine incorporation in response to PHA stimulation is reduced in the presence of SNBTS control buffer early after mitogen stimulation. However, this appears to reflect a delay in lymphocyte transformation rather than true inhibition of the proliferative response.

Citrate present in intermediate purity factor VIII concentrate may contribute to the phenomenon *in vitro* by delaying lymphocyte transformation. Some other component(s) of factor VIII concentrate inhibits lymphocyte proliferation in response to PHA.

The inhibitory substance is unlikely to be factor VIII itself, as a range of high purity concentrates have no effect on ^3H -thymidine incorporation in response to PHA stimulation. Two groups have shown that human factor VIII purified from cryoprecipitate by large-pore gel filtration is not inhibitory to lymphocyte proliferation in response to PHA stimulation (Wang et al, 1985, Daul et al, 1985). Similarly,

monoclonal antibody purified Monoclata is totally devoid of immunosuppressive activity in a PHA stimulation assay at a range of concentrations (Schreiber et al, 1987).

Albumin is unlikely to cause inhibition *in vitro*. Human albumin is included at high concentrations in the preparations Monoclata, Hemofil-M and Monoclata-P as a stabiliser. These concentrates do not inhibit lymphocyte proliferation *in vitro*.

SNBTS salt-poor albumin is reported to be strongly inhibitory to lymphocyte transformation *in vitro* (McDonald et al, 1985). However, following the removal of citrate and other salts by dialysis, this preparation becomes non-inhibitory, supporting the interpretation that albumin plays no part in this phenomenon.

Freshly reconstituted SNBTS factor VIII that had been syringe filtered through a membrane of 0.2µm pore diameter was found to inhibit lymphocyte proliferation in response to PHA. This type of syringe filter should remove protein aggregates. It is therefore unlikely that large protein aggregates are the inhibitory component of SNBTS factor VIII preparations.

Schultz and Shahidi (1990) have recently shown that fibronectin is a potent inhibitor of lymphocyte reactivity *in vitro*, both in purified form and in plasma. The authors demonstrate that purified human fibronectin depresses PHA induced mitogenesis in a concentration-dependent manner over a dose range of 100-400 µg/ml. Similar levels of fibronectin certainly are present when intermediate purity factor VIII concentrates are included in lymphocyte proliferation assays. It is likely that the fibronectin component of factor VIII preparations contributes to inhibition of lymphocyte proliferation in response to PHA *in vitro*.

The effect of purified human fibronectin on lymphocyte activation by PHA should obviously be confirmed using various assay systems eg. ³H-thymidine incorporation, IL2 production and cell surface activation marker expression. If fibronectin is the immunosuppressive component of intermediate purity factor VIII concentrates, then selective removal of fibronectin should also remove the inhibitory activity from immunosuppressive concentrates. This could be achieved by immunoaffinity chromatography using a monoclonal antibody specific for fibronectin. In the present study immunomagnetic isolation was employed in order to deplete SNBTS Z8 factor VIII concentrate of fibronectin. However, the extent to which fibronectin was removed using this approach was not confirmed.

Patients receiving factor IX concentrates appear to have fewer immune abnormalities *in vivo* when compared to haemophiliacs receiving factor VIII concentrates (Lee et al, 1984). In addition, factor IX concentrates have substantially lower fibronectin content compared to intermediate purity factor VIII concentrates. However, SNBTS factor IX concentrate inhibited lymphocyte transformation *in vitro* in response to PHA.

Inhibition may be due to some contaminant other than fibronectin present in factor IX concentrate. It is interesting to note that the concentration of citrate in SNBTS factor IX is approximately three times that in SNBTS factor VIII concentrate.

Inhibition of lymphocyte transformation by SNBTS factor IX concentrate may have been complicated by lectin-glycoprotein interactions. This factor IX preparation has been shown to bind to PHA immobilized on agarose, but not to other mitogen-agarose gels (McDonald et al, 1985). SNBTS factor IX may therefore inhibit lymphocyte proliferation indirectly by reducing the concentration of PHA available for the stimulation of cells. In contrast, SNBTS factor VIII concentrate did not bind to agarose-PHA gels.

Lymphocytes stimulated with PHA and incubated in the presence of intermediate purity factor VIII concentrate express reduced levels of cell surface activation markers CD25 and CD71. This observation has been recently reported by Hay et al (1990), who demonstrate that factor VIII concentrate causes not only reduced numbers of T lymphocytes bearing activation markers, but reduced numbers of activation antigens per cell.

The effect of SNBTS factor VIII on lymphocyte activation marker expression is dose related. Factor VIII does not cause non-specific down regulation of all cell surface molecules as the expression of CD44 remains constant over a range of factor VIII concentrations.

The percentage of cells staining positive for CD25 and CD71 was reduced when SNBTS control buffer was present at a high concentration. This may reflect delayed lymphocyte activation rather than inhibition of cell proliferation. Activation marker expression has been assessed at one time point only.

In accordance with the ^3H -thymidine uptake results, monoclonal antibody-purified factor VIII, Monoclone-P, had no effect on lymphocyte cell surface activation marker

expression. Factor VIII itself and albumin are therefore unlikely to be responsible for reduced cell surface activation marker expression.

The prototype higher purity SNBTS factor VIII concentrate S8 behaved in a similar manner to conventional intermediate purity Z8 concentrate in this assay *in vitro*. SNBTS S8 factor VIII concentrate has significantly lower fibrinogen content compared to SNBTS Z8. It is therefore unlikely that fibrinogen is the protein contaminant responsible for reduced cell surface activation marker expression following PHA stimulation. Although both concentrates have similar citrate contents, SNBTS S8 has an approximately two fold higher fibronectin content compared with SNBTS Z8. Unfortunately, time did not permit the inclusion of SNBTS S8 in a conventional ³H-thymidine uptake assay.

The percentage of cells staining positive for HLA.DR was rather low throughout. It is possible that the time point chosen for cell surface marker analysis was not optimal for HLA.DR expression. Cell surface activation marker expression may well be influenced by the concentration of stimulatory mitogen used, type of culture medium or the concentration of cells cultured. Alternatively, the monoclonal antibody used in the assay may not have had high affinity for HLA.DR. This seems unlikely as initial titration of the monoclonal antibody against a cell line expressing HLA.DR was satisfactory (Data not shown).

Lymphocytes stimulated with PHA and incubated in the presence of intermediate purity factor VIII concentrates produced lower levels of IL2 compared to control cultures. IL2 levels were reduced at both 24 and 48 hours post stimulation. Factor VIII was not directly responsible for reduced ³H-thymidine incorporation by the IL2 dependent cell line as proliferation in response to recombinant IL2 was not affected by the presence of factor VIII. The effect of control buffer on IL2 secretion following PHA stimulation was not investigated.

The inhibitory effect of factor VIII concentrate on IL2 secretion by human T cells has been demonstrated by Lederman et al (1986) and Thorpe et al (1989). The component(s) of factor VIII preparations responsible for reduced IL2 secretion *in vitro* has not yet been identified.

Factor VIII concentrate inhibited lymphocyte proliferation only when present throughout

the culture period. Peripheral blood mononuclear cells incubated with factor VIII overnight and then washed extensively responded normally to PHA. My results contrast with those of Lederman et al (1986) who report that prolonged incubation of lymphocytes with lyophilized factor VIII concentrate resulted in an irreversible inhibition of proliferative responses to PHA. The two studies are not directly comparable as different factor VIII concentrates were investigated under different *in vitro* culture conditions. In particular, the concentration of PHA mitogen used to stimulate lymphocytes was not the same.

The levels of factor VIII required to cause significant inhibition of lymphocyte proliferation *in vitro* in response to PHA were generally high. Lymphocyte proliferation assays in the continued presence of high levels of factor VIII concentrate *in vitro* are therefore somewhat artificial. During factor VIII substitution therapy, levels of factor VIII are high for a brief period before dropping to baseline. The half-life of factor VIII activity for most concentrates is approximately 13 hours (Berntorp and Nilsson, 1988). The half-life values for various contaminant components of factor VIII concentrate are however unknown. The relevance of this type of assay to the situation *in vivo* is therefore questionable.

In summary, intermediate purity factor VIII concentrate appears to inhibit lymphocyte function by interfering with an early activation event which, in turn, leads to reduced cell surface activation marker expression and inhibition of IL2 secretion. The component responsible for this *in vitro* effect has not been identified but fibronectin is a candidate. Citrate contributes to altered lymphocyte function by delaying transformation. High purity factor VIII preparations have no effect on PHA stimulation *in vitro*.

2 SNBTS batch Z8-6026

SNBTS batch Z8-6026 inhibited lymphocyte proliferation in response to PHA when present at high concentrations, but enhanced proliferation over a lower dose range. Similar effects were observed when lymphocytes were stimulated with the recall antigen tetanus toxoid or a suboptimal dose of pokeweed mitogen. SNBTS batch Z8-6026 on

its own did not stimulate lymphocyte proliferation, but seemed to act as a co-mitogen, boosting weak proliferative responses.

This batch of factor VIII was manufactured early in 1987 and was heat treated at 80°C for 72 hours, as is normal. When compared to other batches of Z8 assayed (Appendix I), Z8-6026 has significantly lower fibronectin content. The reduced inhibitory activity of this batch of factor VIII may therefore be related to low fibronectin. Citrate present in the factor VIII formulation may be responsible for delaying lymphocyte proliferation when included at a high concentration.

It is possible that in the absence of inhibitory fibronectin, humoral growth factors, nutrients and cofactors enhance proliferation in response to PHA.

SNBTS Z8-6026 was one of two batches of factor VIII manufactured from 'FB' type plasma. The majority of batches of factor VIII are manufactured from plasma collected and frozen within eight hours ('FA' type plasma). Plasma for SNBTS batches Z8-6026 and Z8-92340 was collected and frozen within eighteen hours. Theoretically, such factor VIII concentrate could contain a higher concentration of degraded proteins or cell surface molecules such as HLA antigens and blood group antigens. However, SNBTS batch Z8-92340 did not enhance lymphocyte proliferation in response to PHA stimulation over a range of concentrations tested. The effect is therefore unlikely to be related to duration of plasma storage.

Lederman et al (1986) fractionated factor VIII concentrate by gel filtration and analysed fractions for inhibitory activity in PHA stimulation assays. It is interesting to note that along with two inhibitory fractions, intermediate fractions from some preparations actually enhanced lymphocyte proliferative responses to PHA. These fractions had no effect on unstimulated lymphocyte cultures.

3 Effects of factor VIII concentrate on lymphocyte proliferation following stimulation with irradiated allogeneic lymphoblastoid cells

A range of factor VIII preparations was assayed in a weak one-way mixed lymphocyte reaction *in vitro*. Lymphocyte proliferation in the presence of allogeneic stimulator cells was correlated with product purity.

All batches of intermediate purity SNBTS Z8 factor VIII assayed enhanced lymphocyte proliferation in this system. Responding cells comprised CD4⁺ and CD8⁺ T cells expressing a range of cell surface activation markers. Prototype higher purity SNBTS S8 and BPL 8Y factor VIII concentrates enhanced the response to a lesser extent. In contrast, all ultra-pure or monoclonal antibody purified products had no effect on proliferation in this assay. Enhanced proliferation was not therefore due to factor VIII itself nor to albumin. Similarly, SNBTS control buffer had no effect on proliferation in response to allogeneic stimulator cells.

Protein aggregates are unlikely to be responsible for enhanced lymphocyte proliferation as the removal of such aggregates by syringe filtering did not effect the co-mitogenic properties of SNBTS factor VIII concentrate.

SNBTS factor IX concentrate enhanced the proliferative response in mixed lymphocyte culture when present at a high concentration. It is unclear whether the co-mitogenic factor(s) present in SNBTS factor IX concentrate is the same as that in SNBTS Z8 and S8 concentrates. The three concentrates do not have equivalent contaminating proteins; for example, SNBTS factor IX concentrate contains very little fibrinogen or fibronectin, proteins which are the main contaminants of SNBTS Z8 and S8 concentrates. The concentrates may however contain a common, minor contaminant, responsible for this phenomenon *in vitro*.

None of the concentrates assayed was mitogenic alone. Some co-mitogenic factor(s) in the preparations boosted a weak proliferative response.

Fractionating SNBTS factor VIII by conventional biochemical means proved to be problematic. The concentrate tended to clot during preparative procedures such as dialysis and during column fractionation. Several basic physical approaches were therefore taken in attempt to characterise the factor(s) responsible for the effects observed *in vitro*.

Freeze-thawing and filtering did not abrogate the co-mitogenic effect. The proliferative response to irradiated allogeneic cells in the presence of defibrinated factor VIII was only slightly reduced. It is unlikely that fibrinogen was completely removed from the factor VIII concentrate. Analysis of 'defibrinated' Z8 factor VIII by SDS-PAGE showed that the protein profile was similar to that of freshly reconstituted factor VIII, but with fewer high molecular weight bands. The addition of thrombin to reconstituted

factor VIII concentrate not only accelerates the formation of fibrin from fibrinogen monomers, but activates factor VIII.

The contaminant responsible for enhanced proliferation in response to allogeneic stimulator cells is unlikely to be fibrinogen. SNBTS factor IX and SNBTS S8 prototype factor VIII concentrates contain only low levels of fibrinogen but enhanced proliferation in weak one-way mixed lymphocyte cultures.

SNBTS Z8 factor VIII concentrate was filtered through Amicon ultra filtration devices in order to fractionate the constituents into broad molecular weight bands. The enhancing activity appeared to be confined to the fraction containing molecules of 100 KDa or over. However, SDS-PAGE analysis revealed the factor VIII had not been fractionated efficiently. The protein profile of the '>100 KDa fraction' was identical to that of the unfractionated defibrinated factor VIII. The '>100 KDa fraction' contained molecules of <100 KDa. Similarly, traces of high molecular weight molecules are apparent in the '<100 KDa fraction'. In addition, the protein concentration in the '<100KDa' fraction is very much lower than for defibrinated factor VIII or the '>100 KDa fraction'. The lack of enhanced cell proliferation by the '<100 KDa fraction' may therefore be due to dilution, rather than to absence of the factor responsible.

Defibrinated factor VIII was filtered through Amicon ultrafiltration devices with molecular weight cut off values of 30 KDa and 10 KDa. When assayed *in vitro* the enhancing activity was confined to the >30 KDa fraction. As SDS-PAGE analysis of the fractions obtained was not performed, it is unclear if the fractions assayed *in vitro* contained molecules only within the molecular weight range stated.

Klingemann et al (1986) demonstrated that fibronectin restored defective lymphocyte proliferation *in vitro* in the mixed lymphocyte reaction of immunocompromised bone marrow transplant patients. Fibronectin and monoclonal antibodies specific for CD3 have also been shown to be costimulants of human CD4 positive lymphocyte proliferation under appropriate culture conditions (Matsuyama et al, 1989, Shimizu et al, 1990). It therefore seemed plausible that fibronectin, present in factor VIII concentrates, may play a role in enhancing the mixed lymphocyte reaction *in vitro*. However, a range of concentrations of purified fibronectin were assayed *in vitro* and found to be completely devoid of co-mitogenic activity.

Three protein contaminants present in SNBTS factor VIII were targeted for removal by immunomagnetic isolation. Monoclonal antibodies to HLA.DR, fibronectin and factor VIII/von Willebrand factor were immobilized on magnetic particles and incubated with factor VIII concentrate. The resulting 'depleted' samples were included in a weak one-way mixed lymphocyte reaction. All enhanced lymphocyte proliferation to the same extent as untreated factor VIII concentrate. These three proteins may not therefore be responsible for the observed effect. However, these results are preliminary; the efficiency of protein depletion using this approach has not been confirmed.

It is not clear why some component of factor VIII enhances lymphocyte proliferation in the presence of a weak allogeneic stimulus.

Matheson et al (1986) observed a positive blastogenic response to low concentrations of lyophilized factor VIII concentrate in a seven day thymidine uptake assay. However, responses were fairly weak and only occurred in haemophilia patients who had previously been exposed to factor VIII concentrate. Peripheral blood mononuclear cells from healthy controls did not respond to the factor VIII concentrate *in vitro*. The presence of T cells primed to factor VIII in haemophilia patients has also been suggested by Madhok et al (1991).

In contrast, I have observed that Scottish factor VIII enhanced the proliferative response of non-haemophiliac cells to irradiated allogeneic lymphoblastoid cells. Responder lymphocytes had not previously been exposed to factor VIII concentrate and could not therefore be considered antigenically primed to some constituent of SNBTS Z8 concentrate.

B cells can process antigen efficiently and present the internalized derivative (Pierce et al, 1988). It is therefore possible that the irradiated allogeneic lymphoblastoid B cells are presenting some antigen(s) in the factor VIII concentrate. This seems unlikely in view of the fact that allogeneic cells incubated with factor VIII overnight, then extensively washed, do not enhance the mixed lymphocyte reaction *in vitro*. Similarly, allogeneic cells incubated with factor VIII for four hours prior to fixation with paraformaldehyde do not stimulate well *in vitro*. In contrast, fixed or irradiated lymphoblastoid cells incubated with factor VIII concentrate throughout the culture period are strongly stimulatory to peripheral blood mononuclear cells.

Alloreactivity *in vitro* is controlled by polymorphic class II molecules encoded in the major histocompatibility complex (Eckels D D, 1989). Cellular immune responses in the mixed lymphocyte reaction are very much dependent on stimulator cell HLA.DR expression. The expression of HLA.DR antigen by the lymphoblastoid stimulator cell line was therefore investigated in the presence or absence of intermediate purity factor VIII concentrate. However, SNBTS factor VIII concentrate did not alter the level of expression of HLA.DR antigen. Increased HLA.DR expression did not contribute to enhanced cell proliferation *in vitro* in the presence of factor VIII.

It is probable that the observed *in vitro* effect of factor VIII concentrate in this assay is related to the availability of nutrients and growth factors. Peripheral blood mononuclear cells and irradiated lymphoblastoid cells remained viable for a longer period of time when incubated in medium supplemented with factor VIII concentrate in addition to heat inactivated foetal calf serum and fresh glutamine.

When mixed lymphocyte reactions were performed in medium supplemented with autologous plasma, peripheral blood mononuclear cells responded well to irradiated lymphoblastoid cells and neither SNBTS factor VIII nor control buffer affected the response in any way. The co-mitogenic effect of factor VIII concentrate on proliferation in response to allogeneic cells is only apparent under suboptimal culture conditions. It seems likely that some substance present both in intermediate purity factor VIII concentrate and in normal plasma is responsible for the effect observed *in vitro*.

My results contrast those of Lederman et al (1986) who reported reduced lymphocyte proliferation in response to allogeneic stimulator cells in the presence of lyophilized factor VIII concentrate. It is difficult to compare these conflicting results as no technical details of culture conditions used by the previous authors are available. In addition, Lederman et al did not include NHS concentrate manufactured in the United Kingdom but assayed three commercial American products.

4 Lymphocyte proliferation, factor VIII and HIV infection

Peripheral blood mononuclear cells from a healthy individual were infected with a laboratory strain of HIV and included in proliferation assays *in vitro*.

Lymphocyte proliferation in response to PHA was inhibited by SNBTS Z8 factor VIII concentrate in a dose-dependent fashion. HIV replication, as assessed by p24 antigen production approximated to lymphocyte proliferation. Similarly, p24 antigen production correlated with lymphocyte proliferation in response to allogeneic stimulator cells and intermediate purity factor VIII concentrate. It is not surprising that HIV replication was related to lymphocyte proliferation as HIV is known to preferentially infect, and replicate in activated T4 lymphocytes (Margolick and Fauci, 1987). It must be stressed that no virus replication occurred in the presence of SNBTS factor VIII alone. As in non HIV-infected lymphocytes, proliferation in response to allogeneic stimulator cells was boosted in the presence of factor VIII.

I have demonstrated that intermediate purity factor VIII concentrate may modulate immune reactivity and therefore HIV virus replication *in vitro*. Intermediate purity factor VIII concentrate could potentially affect immune reactivity *in vivo*. PHA is not a physiological stimulus but a non-specific T cell mitogen. The relevance of such stimulation *in vitro* to the situation *in vivo* is therefore doubtful. In contrast, lymphocyte proliferation in the mixed lymphocyte reaction *in vitro* is based on the phenomenon of allograft rejection *in vivo* and may therefore be a more relevant experimental model. If this is so, then the possibility arises that infusions of factor VIII may enhance lymphocyte activation in response to naturally occurring immune stimuli encountered *in vivo*. In an HIV antibody positive haemophilia patient this could lead to increased virus replication and thus accelerated HIV disease progression. To date, there has been no conclusive evidence to support a role for intermediate purity factor VIII in accelerating HIV disease progression.

The effect of intermediate versus high purity factor VIII concentrate on HIV disease progression is unresolved. Some groups claim high purity factor VIII preparations stabilise immune parameters in HIV antibody positive haemophilia patients (Fukutake et al, 1991, de Biasi et al, 1991, Goldsmith et al, 1991). Others however, do not support a beneficial role for high purity concentrates in the treatment of HIV antibody positive haemophilia patients (Mannucci et al, 1991, Transfusion Safety Study [TSS] Group, 1991).

Most studies have been in progress for short periods of time and may be more informative in the future.

In studies of this type it is essential that patient groups are correctly balanced. In particular, patients should be matched in terms of HIV disease stage, blood product usage and age, before being assigned to receive treatment with intermediate or high purity factor VIII concentrate. The presence and severity or absence of liver disease should also be considered in such prospective trials.

The response to treatment with high purity factor VIII concentrates may vary depending on how far HIV disease has progressed. Patients may fare better on high purity factor VIII products if they receive them early in the course of HIV disease, while their immune system is relatively intact.

Unfortunately, it is impossible to investigate the effect of intermediate purity SNBTS factor VIII concentrate on HIV disease progression in the Edinburgh haemophilia cohort, as the group comprises only eighteen patients. A strong effect from the HLA haplotype A1 B8 DR3 on HIV disease progression in this group of patients further confounds such analysis.

5 Lymphocyte proliferation in haemophilia patients

The effect of SNBTS Z8 factor VIII concentrate on the proliferation of peripheral blood mononuclear cells from haemophilia patients was investigated. However, as numbers were extremely limited, the conclusions drawn are preliminary and should be confirmed.

Immune reactivity to allogeneic stimulator cells was not impaired in HIV antibody negative haemophilia patients. ³H-thymidine incorporation in response to irradiated lymphoblastoid cells in medium supplemented with autologous plasma was comparable to that found for non-haemophiliac bloods and was not enhanced by the presence of SNBTS Z8 factor VIII concentrate. Haemophilia patients are not therefore immunosuppressed with respect to alloantigens.

Mixed lymphocyte reactions using lymphocytes from haemophilia patients were not conducted in medium containing heat inactivated foetal calf serum. It would obviously be interesting to determine whether SNBTS factor VIII enhances lymphocyte proliferation under these assay conditions.

The behaviour of lymphocytes from haemophilia patients infected with HIV in one-way mixed lymphocyte reactions *in vitro* was abnormal. Responses to irradiated allogeneic stimulator cells were extremely poor and only marginally improved in the presence of SNBTS factor VIII. The mixed lymphocyte reaction appeared to peak lower, early and for a limited period of time. The response to allogeneic stimulator cells was not sustained in HIV positive haemophilia patients.

In general, the response to allogeneic stimulator cells *in vitro* peaks around day five or six of culture. In the HIV positive patients however, ^3H -thymidine incorporation in the one-way mixed lymphocyte reaction typically declined from day three.

^3H -thymidine incorporation was not due to lymphocytes pre-activated *in vivo* nor to lymphoblastoid cells as both these cell types did not proliferate when cultured alone. In the presence of SNBTS Z8 factor VIII concentrate lymphocytes may remain viable and responsive to allogeneic stimulation for a short period, thus causing a small increase in ^3H -thymidine incorporation. SNBTS control buffer and monoclonal antibody purified factor VIII did not affect ^3H -thymidine incorporation in this assay.

Both haemophilia patients studied had been infected with HIV for over seven years and displayed all the immunological characteristics of HIV disease progression. Patients had low CD4^+ T lymphocyte counts, high numbers of activated T cells and high levels of circulating soluble IL2-receptor and serum β_2 microglobulin.

Virus proliferation *in vitro* was measured in terms of p24 antigen production. p24 antigen levels in one patient remained below the lower threshold of the assay. In contrast, p24 was readily detectable in cell supernatants from patient (4). The p24 levels did not correlate well with ^3H -thymidine incorporation. Although the concentration of p24 appears to be low, it should be noted that the virus assayed originates from only 5×10^4 peripheral blood mononuclear cells. p24 antigen production was presumably due to activation of cellular HIV provirus and not a result of cell reinfection from autologous plasma. All plasma used to supplement culture medium was heat inactivated prior to inclusion in the assay.

p24 antigen levels appear to increase in the presence of SNBTS Z8 factor VIII concentrate, SNBTS control buffer and monoclonal antibody purified factor VIII. This type of experiment should obviously be repeated in order to ascertain whether this rise is reproducible or an artifact.

Neither HIV antibody negative nor HIV antibody positive haemophilia patients appeared to be antigenically primed to any component of SNBTS factor VIII concentrate as peripheral blood mononuclear cells incubated with factor VIII concentrate did not proliferate. *In vitro* immune reactivity to factor VIII concentrate has only rarely been reported in haemophilia patients (Matheson et al, 1986, Madhok et al, 1991).

In conclusion, I have confirmed the immunomodulatory nature of factor VIII preparations *in vitro*. Immunomodulatory effects were observed using therapeutic concentrations of factor VIII. The effects were related to the degree of purity of the factor VIII preparation assayed. Factor VIII itself was not responsible for immunomodulation in proliferation assays *in vitro* and the effects were probably not due to fibronectin.

Effects on lymphocyte proliferation were still demonstrable in defibrinated SNBTS Z8 intermediate purity factor VIII concentrate, which was more amenable to physico-chemical manipulation than the reconstituted preparation used for injection. Initial attempts to define the molecular weight of the relevant contaminant responsible for immune modulation *in vitro* were not successful.

Biological effects on lymphocyte proliferation *in vitro* were not observed in the presence of 10% pooled (AB) human serum or heat treated autologous plasma. The co-mitogenic contaminant present in factor VIII preparations may be a component of normal human serum not found in foetal calf serum.

In order to clarify the relationship between the contaminant(s) responsible for immune modulation *in vitro* and immunological abnormalities observed in haemophilia patients treated with intermediate purity factor VIII products *in vivo*, the co-mitogenic activity must first be further chemically characterised. This could involve for instance, ultracentrifugation, to crudely separate components of the concentrate. Specific degradative enzymes such as proteases could also be used to characterise the component. Alternatively, affinity columns could selectively remove specific components of immunomodulatory preparations such as stimulatory cytokines, if present. More subtle fractionation procedures such as high pressure liquid chromatography (HPLC) will probably have to await removal of major non-contributing components (eg. by ultracentrifugation).

When identified the candidate component should be selectively removed from commercial factor VIII preparations. This could be achieved by immunoaffinity chromatography. The depleted concentrates should not modulate immune activity *in vitro*. Factor VIII concentrates lacking the identified immunomodulatory component should then be tested *in vivo*. Patient immune status (eg. reactivity to skin test antigens) should be compared before and after receiving factor VIII concentrate without the specific component. If the identified component is indeed responsible for immune modulation, then immune function in patients receiving this material should show improved immunological status *in vivo*. In addition, patients treated entirely with such a depleted preparation should not develop any of the immunological abnormalities associated with the use intermediate purity factor VIII concentrates for the treatment of haemophilia.

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APPENDIX I

Appendix I lists batches of SNBTS Z8 factor VIII included in this study. All product information presented was kindly provided by the SNBTS Protein Fractionation Centre, Edinburgh.

APPENDIX I (i)

BATCH NUMBER	TOTAL PROTEIN g/l	FIBRINOGEN g/l	FIBRONECTIN g/l	FACTOR VIII:C 1 STAGE ASSAY iu/ml	FACTOR VIII:C 2 STAGE ASSAY iu/ml	FACTOR VIII PER VIAL iu
Z8-6026	23.0	7.60	1.92	10.04	6.26	208.7
Z8-81561	22.0	7.25	4.80	9.76	6.06	203.0
Z8-81562	22.4	9.30	4.70	10.31	6.39	214.4
Z8-81760	18.9	6.80	2.76	7.61	3.57	158.2
Z8-81770	23.8	9.30	3.90	11.04	7.50	229.6
Z8-91930	21.6	8.20	4.30	9.69	7.08	201.5
Z8-92110	24.5	7.70	7.20	9.94	7.97	206.7
Z8-92210	24.3	11.9	5.20	10.72	7.20	222.9
Z8-92250	23.3	10.3	5.30	11.09	6.69	230.6
Z8-92280	25.1	12.5	6.10	9.96	6.18	207.1
Z8-92300	22.6	9.10	5.50	9.96	6.23	207.1
Z8-92340	26.7	10.9	7.60	11.31	7.89	235.2
Z8-92360	23.5	8.30	4.20	10.72	8.43	222.9
Z8-92380	24.0	9.20	4.70	12.25	7.93	254.8
Z8-92430	23.5	10.7	5.30	9.69	7.98	201.5
Z8-92450	24.6	12.1	5.00	9.25	8.22	192.4
Z8-92490	22.7	10.9	5.10	11.91	9.32	247.7
Z8-92520	22.5	10.1	6.00	9.92	7.77	206.3
Z8-02560	24.4	10.2	6.30	11.59	10.03	241.0
MEAN	23.3 +/- 1.6	9.60 +/- 1.68	5.05 +/- 1.35	10.36 +/- 1.07	7.15 +/- 1.28	215.3 +/- 22.2

APPENDIX I (ii)

BATCH NUMBER	SODIUM mmol/l	CHLORIDE mmol/l	ZINC ppm	CALCIUM ppm	ALUMINIUM ppb	CITRATE mmol/l	SUCROSE %
Z8-6026	156	97.5	10.00	144.0	55.0	24.2	2.31
Z8-81561	157	106.5	4.10	115.0	74.4	22.5	2.24
Z8-81562	157	109.5	6.40	110.0	35.1	23.9	2.31
Z8-81760	142	95.5	6.10	106.0	46.3	21.6	2.10
Z8-81770	150	103.0	8.30	106.0	50.2	21.2	2.16
Z8-91930	148	98.0	10.50	136.0	81.5	21.3	2.47
Z8-92110	150	101.5	10.70	112.5	47.8	21.3	2.03
Z8-92210	153	105.0	7.50	112.5	53.1	22.5	2.07
Z8-92250	152	111.0	4.75	130.0	55.3	23.0	2.14
Z8-92280	152	95.5	5.25	122.5	101.0	22.7	2.15
Z8-92300	154	97.0	5.40	115.0	55.3	21.0	2.15
Z8-92340	148	105.0	5.50	130.0	84.6	21.0	2.02
Z8-92360	151	102.0	6.90	125.0	78.0	21.6	2.02
Z8-92380	148	100.0	5.50	112.5	81.3	20.7	2.02
Z8-92430	151	101.0	8.90	125.0	76.0	21.2	2.01
Z8-92450	149	99.0	9.75	120.0	83.0	20.9	2.02
Z8-92490	151	103.0	6.38	136.5	115.0	22.4	2.01
Z8-92520	156	109.0	7.00	140.0	113.0	22.4	2.19
Z8-02560	151	102.5	7.75	131.0	48.0	21.6	2.12
MEAN	151 +/- 4	102 +/- 5	7.19 +/- 2.01	122.5 +/- 12	70.2 +/- 23.2	21.9 +/- 1	2.13 +/- 0.13

APPENDIX I (iii)

BATCH NUMBER	ENDOTOXIN iu/ml	PYROGEN 'C/3 rabbits	MOISTURE %	pH	PLASMA TYPE†	SOLUBILITY mins	OSMOLALITY mOsm/kg
Z8-6026	<1.250	0.3	0.52	6.88	FB	2.5	338
Z8-81561	<0.500	0.2	1.19	6.97	FA	5.0	344
Z8-81562	<0.500	0.4	1.23	7.10	-	5.0	334
Z8-81760	0.500-1.000	0.8	1.26	7.03	FA	5.0	300
Z8-81770	<0.500	0.6	1.05	7.06	FA	5.0	318
Z8-91930	1.750-2.500	0.7	1.06	6.89	FA	5.0	316
Z8-92110	0.500-1.000	0.3	1.03	6.86	FB	5.0	323
Z8-92210	0.500-1.000	0.3	1.14	6.93	FA	5.0	330
Z8-92250	<0.500	0.2	1.45	6.89	FA	5.0	330
Z8-92280	<0.125	0.3	1.75	6.87	FA	5.0	324
Z8-92300	0.500-1.000	0.5	1.63	6.90	FA	5.0	323
Z8-92340	<0.125	0.3	1.09	6.94	FB	5.0	317
Z8-92360	<0.125	0.1	1.40	6.82	FA	5.0	328
Z8-92380	<0.125	0.8	1.31	6.89	FA	5.0	322
Z8-92430	<0.125	1.0	1.14	6.89	FA	5.0	324
Z8-92450	<0.125	1.1	1.08	6.87	FA	5.0	325
Z8-92490	<0.125	0.4	0.73	6.94	FA	5.0	334
Z8-92520	<0.125	0.6	0.71	6.95	FA	5.0	342
Z8-02560	<0.500	0.2	0.86	6.88	FA	5.0	313
MEAN	-	0.5 +/- 0.3	1.14 +/- 0.31	6.92 +/- 0.07	-	5.0 +/- 0.6	326 +/- 11

† FA; plasma collected and frozen within 8 hours
FB; plasma collected and frozen within 18 hours

APPENDIX II

Appendix II summarises total protein concentration, factor VIII activity and mode of virus inactivation for concentrates included in this study. Product information presented is as stated in manufacturers' product insert or was provided by the SNBTS Protein Fractionation Centre, Edinburgh.

APPENDIX II

FACTOR VIII	TOTAL PROTEIN g/l	FACTOR VIII:C iu/ml	VIRUS INACTIVATION
SNBTS	23.3+/-1.6	10.36+/-1.07	DRY HEAT 80 °C 72 hours
BPL 8Y	6.0+/-1.0	23.5+/-3.1	DRY HEAT 80 °C 72 hours
PROFILATE HEAT-TREATED	5	22	WET HEAT TREATED 60 °C 20 hours
OCTA-PHARMA V.I.250	5	25	SOLVENT-DETERGENT
BIO-TRANSFUSION	0.24+/-0.085	42.5+/-9.5	SOLVENT-DETERGENT
HEMOFIL-M	-	31.1	SOLVENT-DETERGENT
MONOCLATE	7.5+/-2.5	100	DRY HEAT 60 °C 30 hours
MONOCLATE-P	7.5+/-2.5	100	PASTEURIZED 60 °C 10 hours

APPENDIX III

Appendix III compares SNBTS Z8, SNBTS S8 and SNBTS factor IX concentrates.

Product characteristics were kindly provided by the SNBTS Protein Fractionation Centre, Edinburgh.

APPENDIX III

ASSAY	UNIT	SNBTS Z8 (mean)	SNBTS S8 0006	SNBTS FIX 90560
total protein	g/l	23.3+/-1.6	15.5	16.2
fibrinogen	g/l	9.60+/-1.68	4.2	0
fibronectin	g/l	5.05+/-1.35	10.8	-
sodium	mmol/l	151+/-4	193	232.0
chloride	mmol/l	102+/-5	116	1.00
citrate	mmol/l	21.9+/-1	27.5	59.2
pH		6.92+/-0.07	7.14	7.11
sucrose	%	2.13+/-0.13	6.04	-
moisture	%	1.14+/-0.31	0.7	-
zinc	ppm	7.19+/-2.01	7.75	-
calcium	ppm	122.5+/-12	115.0	-
osmolality	mOsm/kg	326+/-11	522	-
aluminium	ppb	70.2+/-23.2	258	-
VIII:C 1	iu/ml	10.36+/-1.07	20.24	-
VIII:C 2	iu/ml	7.15+/-1.28	18.42	-
potassium	mmol/l	-	0.11	0.08
phosphate	mmol/l	-	-	36.9
F IX	iu/ml	-	-	37.07
Xa generation	min	-	-	25
NAPTT	sec	-	-	304
F II	iu/ml	-	-	58.38
F X	iu/ml	-	-	35.71

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